UNIVERSIDAD AUTÓNOMA DE MADRID
FACULTAD DE CIENCIAS
DEPARTAMENTO DE BIOLOGÍA

DOCTORAL THESIS
Biology PhD

"Effect of a caloric restriction based on the Mediterranean diet and intake of traditional Mediterranean foods on the expression of microRNAs regulating molecular processes associated with aging"

INSTITUTO MADRILEÑO DE ESTUDIOS AVANZADOS EN ALIMENTACIÓN (IMDEA FOOD INSTITUTE)

VÍCTOR MICÓ MORENO

Madrid, 2018

UNIVERSIDAD AUTÓNOMA DE MADRID
FACULTAD DE CIENCIAS
DEPARTAMENTO DE BIOLOGÍA

DOCTORAL THESIS
Biology PhD

"Effect of a caloric restriction based on the Mediterranean diet and intake of traditional Mediterranean foods on the expression of microRNAs regulating molecular processes associated with aging"

INSTITUTO MADRILEÑO DE ESTUDIOS AVANZADOS EN ALIMENTACIÓN (IMDEA FOOD INSTITUTE)

Memoria presentada por: **Víctor Micó Moreno**
Para optar al grado de: DOCTOR EN BIOLOGÍA

Doña Lidia Ángeles Daimiel Ruíz, Doctora en Biología Celular y Genética por la Universidad Autónoma de Madrid, investigadora del Instituto IMDEA Alimentación, informa favorablemente la solicitud de autorización de defensa de la tesis doctoral con el Título: **"Effect of a caloric restriction based on the Mediterranean diet and intake of traditional Mediterranean foods on the expression of microRNAs regulating molecular processes associated with aging"**, presentada por Don **Víctor Micó Moreno** para optar al grado de Doctor en Biología. Este trabajo ha sido realizado en el Instituto Madrileño de Estudios Avanzados en Alimentación (IMDEA Alimentación) bajo su dirección, y cumple satisfactoriamente las condiciones requeridas por el Departamento de Biología de la Universidad Autónoma de Madrid para optar al Título de Doctor.

Ha actuado como tutor académico, y presenta su conformidad el Dr. Carlos Francisco Sentís Castaño, vicedecano de Personal Docente e Investigador y profesor titular del Departamento de Biología de la Facultad de Ciencias de la Universidad Autónoma de Madrid.

Y para que así conste, firman el presente informe:

Lidia Ángeles Daimiel Ruiz
Investigadora Junior
Nutritional Genomics and
Epigenomics Group
Precision Nutrition and Obesity
IMDEA Alimentación

Carlos Francisco Sentís Castaño,
Vicedecano de Personal Docente
e Investigador
Profesor Titular
Departamento de Biología
Facultad de Ciencias Universidad
Autónoma de Madrid.

Esta tesis ha sido realizada gracias a la financiación obtenida a través del Instituto de Salud Carlos III (FIS/AES PI14/01374 and PI17/00508), XIV edición de las Becas de Investigación Manuel de Oya otorgadas por la Fundación Cerveza y Salud, Ayudas Merck Serono de Investigación otorgadas por la Fundación Salud 2000 además de un contrato en IMDEA Alimentación financiado por fondos FEDER europeos así como fondos de la Comunidad Autónoma de Madrid.

La realización de la estancia internacional de investigación ha sido financiada por Short-Term Scientific Mission program de POSITIVe Cost Action y por Exchange Award de The European Foundation for Alcohol Research "ERAB".

"Nada en la vida debe temerse,

solo debe ser entendida"

Marie Curie

A mis padres Paco y Amparo

A mi hermana Sara

A Laura

Durante la realización de esta tesis doctoral ha habido muchas personas con las que he compartido parte o todo el camino hasta llegar a su final, con los que he tenido la suerte de compartir muchísimas experiencias y, a los que esta tesis, y lo que ello ha significado, pertenece también y me gustaría recordar.

A la Dra. Lidia Daimiel, directora de esta tesis, y a la que puedo considerar mi mentora en el mundo de la ciencia. Desde el año 2012, momento en el que entré en IMDEA alimentación realizando prácticas extracurriculares de la universidad bajo su tutoría, comenzamos un camino de 6 años que ha resultado en esta tesis. Gracias por acompañarme en el mundo de la ciencia y el apoyo y confianza depositado en mí.

Al Prof. José María Ordovás, director del departamento, el cuál ha sido un placer trabajar en su grupo, y aprender de él.

A todo PREDITEAM, Laura B., Javi, Laura D. y Paloma, sin vuestra ayuda y apoyo no hubiera sido posible presentar esta tesis, que también pertenece a vosotros.

A Ruth, gracias por todo tu apoyo porque, aunque nos conocemos sólo desde hace un año y medio, me llevo una amistad, muchos buenos momentos y el programa “amadrina un becario”.

A todos los estudiantes a los que he tenido la suerte de enseñar, porque también he aprendido mucho de vosotros.

A todo el equipo de medicina y enfermería y administración de los centros de atención primaria que han participado en nuestros estudios, así como a todos los voluntarios.

A todas las personas que tuve la suerte de conocer durante mi estancia en el King´s College London, pero en especial a la Dra. Ana Rodríguez Mateos por acogerme en su grupo y a “Los científicos”: Geoffrey, Paul y Michele. Thanks for all good moments in London guys!

A todos los imdea@s, especialmente Mónica, Su, Silvia, Lara, Cris, Josune, María, Jorge y a todos los predocs que hemos compartido alegrías y penas durante la tesis, por todas las horas compartidas, todos los buenos momentos, todos los misterios e historias ocurridas en IMDEA tratados de ser descubiertos y, en definitiva, haber sido una segunda familia.

A mis amig@s de CYTA, Ana, Laura, Mario, Miguel, Isa y Cris, por los momentos compartidos y por los momentos que aún nos queda por compartir.

A todos mis compañeros “inmigrantes” del máster, pero en especial a Patri y Elena, por todos los buenos momentos y por finalmente entender que es la p en estadística.

A Raquel, de Nutrición, por cumplir aquello que decíamos cuando estudiábamos ¿te imaginas que terminamos doctorándonos?

A mis padres, Paco y Amparo, ya que todo lo que ha sucedido ha sido gracias a ellos y nunca habéis dejado de darme vuestro apoyo incondicional y a mi hermana Sara, que me has marcado el camino que yo he continuado y que nunca has dejado de apoyarme y confiar en mí.

Y por último, me gustaría agradecer y dedicar esta tesis a Laura ya que esta tesis no hubiera sido posible sin todo su apoyo, cariño y comprensión. Gracias por ser mi compañera tanto en este camino como en la vida y estar siempre a mi lado tanto en los buenos como en los malos momentos

INDEX

ABSTRACT / RESUMEN

The demographic trend in Europe shows a gradual aging of the population. Aging is a process that have been associated with the development of age-related diseases as diabetes, cardiovascular disease or cancer. Immune defeat, cognitive impairment or the loss of proper regulation of sensory pathways of metabolic state such as IGF-1/PI3K/AKT/FOXO and AMPK/SIRT1/mTOR pathways are other hallmarks of aging. Aging is an irreversible process, but it can be modulable, and it is also affected by environmental factors as diet. In this sense, a healthy dietary pattern as Mediterranean Diet or its specific foods and caloric restriction have been postulated as the most important interventions that allow increasing the expectancy and quality of life. MicroRNAs, short non-coding RNA sequences are key epigenetic regulators of these nutrient sensing pathways. This thesis is focused in the nutritional modulation of microRNAs related to cardiovascular disease and aging and regulating nutrient sensing pathways. For this purpose, we carried out different intervention studies with functional extra virgin olive oil enriched with its own polyphenols in healthy patients (VOHF study), beer and non-alcoholic beer in volunteers at high cardiovascular risk (miRoBeer study) and Mediterranean Diet (MD) with/without caloric restriction in late adults with metabolic syndrome (PREDIMED-PLUS study). In addition, it has been tested the presence of exogenous microRNAs in human plasma. The results obtained in this thesis showed first, the non-detection of exogenous microRNAs in human plasma in nor in in olive oil and beer. With the VOHF study, we observed that functional extra virgin olive oil consistently regulated let-7e, microRNA implicated in insulin signaling and inflammation, miR-17-92 cluster, which is a regulator of IGF-1/PI3K/AKT/FOXO and AMPK/SIRT1/mTOR pathways and miR-328, implicated in hypertension and cardiovascular disease. In miRoBeer study we observed a downregulation after non-alcoholic beer intake and an upregulation after beer intake of macrophages miR-17-92 cluster, miR-26b, miR-145, miR-223 and circulating levels of miR-17-92 cluster, miR-155 and a downregulation of miR-320 in both types of beer suggesting an anti-inflammatory microRNA profile but a worse glucose homeostasis. In additions, we observed a correlation between macrophage and circulating levels of miR-328, let-7e, miR-92 and miR-26b suggesting a link between both. Last, we observed a downregulation after 1 year of treatment with a hypocaloric Mediterranean diet with physical activity of macrophage miR-130a and miR-30c in MD in PREDIMED-PLUS study and a lower population of senescent lymphocytes. To conclude, we suggest that one of the mechanisms by MD and their associated foods have beneficial effects on human health could be the modulation of microRNAs and the observed modulation of circulating microRNAs suggest the detection of putative biomarkers of nutritional response, but further studies are necessary to confirm this effect.

Europa muestra un envejecimiento gradual de la población que tiene asociado el desarrollo de enfermedades como la diabetes, las enfermedades cardiovasculares o el cáncer además de la disfunción inmune, el deterioro cognitivo o la pérdida de la regulación de vías sensoras del estado metabólico, como la vía de IGF1/PI3K/AKT/FOXO y la vía de AMPK/SIRT1/mTOR. El envejecimiento es un proceso irreversible, pero que a su vez puede ser modulable, y que está afectado por factores ambientales como la dieta. En este sentido, un patrón dietético saludable como la dieta mediterránea o sus alimentos típicos y la restricción calórica se han postulado como algunas de las intervenciones más importantes que permiten aumentar la calidad y esperanza de vida. Los microRNAs, secuencias cortas de ARN no codificantes, son reguladores epigenéticos clave de estas vías sensoras de nutrientes. Esta tesis está centrada en la modulación de microRNAs relacionados con las enfermedades cardiovasculares, envejecimiento y vías sensoras de nutrientes. Para ello, hemos realizado diferentes estudios de intervención con aceite de oliva virgen extra funcional enriquecido en sus propios polifenoles en pacientes sanos (estudio VOHF), un estudio con cerveza y cerveza sin alcohol en voluntarios con alto riesgo cardiovascular (estudio miRoBeer) y un estudio con Dieta Mediterránea (DM) con/sin restricción calórica en adultos de edad avanzada con síndrome metabólico (estudio PREDIMED-PLUS). Además, se ha testado la presencia de microRNAs exógenos en plasma humano. Los resultados obtenidos en esta tesis muestran, en primer lugar, la no detección de microRNAs exógenos en plasma humano así como en aceite de oliva y cerveza. En el estudio VOHF observamos que el aceite de oliva virgen extra funcional regulaba consistentemente let-7e, microRNA implicado en la cascada de señalización de la insulina e inflamación, miR-17-92 cluster, regulador del IGF-1/PI3K/AKT/FOXO y AMPK/SIRT1/mTOR y miR-328, implicado en la hipertensión y las enfermedades cardiovasculares. En el estudio miRoBeer se observó una sobreexpresión en cerveza con alcohol y una represión en cerveza sin alcohol del miR-17-92 cluster, miR-26b, miR-145, miR-223 en macrófagos y en los niveles circulantes de miR-17-92 cluster, miR-155 siendo una represión de miR-320 en ambos tipos de cerveza, sugiriendo un perfil de microRNAs antiinflamatorio pero con una peor homeostasis de la glucosa. Además, observamos correlaciones entre los niveles de macrófagos y circulantes de miR-328, let-7e, miR-92 y miR-26b, sugiriendo un vínculo entre ambos. Por último, observamos una represión de miR-130a y miR-30c después de 1 año de tratamiento con DM con restricción calórica en el estudio PREDIMED-PLUS, además de una menor población de linfocitos senescentes. Por todo ello sugerimos que uno de los mecanismos por los que la DM y sus alimentos asociados tienen efectos beneficiosos en la salud humana podría ser la modulación de los microRNAs además de que la modulación observada de microRNAs circulantes sugiere la detección de biomarcadores de respuesta nutricional, aunque son necesarios más estudios para confirmar este efecto.

ABBREVIATIONS

8-PG: 8-Prenilnarigerin

AF: atrial fibrillation

AGO: Argonaute proteins

AHL: Age-related hearing loss

AMI: acute myocardial infarction

AMP: Adenosine monophosphate

AMPK: AMP-activated protein kinase

APOE: Apolipoprotein E

ATP: Adenosine triphosphate

BAT: Brown adipose tissue

BMDMs: Bone marrow-derived macrophages

BMI: Body mass index

CAD: Coronary artery disease

CALERIE: Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy

CHD: Coronary heart disease

CMVEC: Cerebrovascular endothelial cells

CR: Caloric restriction

CRON: Calorie Restriction with Optimum Nutrition

CRP: C reactive protein

CVD: Cardiovascular disease

DHA: docosahexaenoic acid

DMSO: Dimethyl sulfoxide

ECG: Electrocardiogram

EDTA: Ethylenediaminetetraacetic acid

EVOO: Extra virgin olive oil

FBS: Fetal bovine serum

FEAST: Alternate day fasting in humans

FFQ Food consumption frequency questionnaire

FOXO: Forkhead Box O

GDM: Gestational Diabetes Mellitus

GCN2: General control nonderepressible 2

GH: Growth hormone

GTP: Guanosine triphosphate

HDL: High density lipoprotein

ICM: Ischemic cardiomyopathy

IF: Intermittent Fasting

IGF1: Insulin-like growth factor-1

INFgamma: Interferon Gamma

IX: Isoxanthohumol

KO: Knock out

LDL: Low density lipoprotein

LDLR: Low density lipoprotein receptor

LDD: lumbar disc degeneration

LPS: Lipopolysaccharides

MD: Mediterranean Diet

MMSE: Mini-Mental State Examination

mTOR: Mammalian target of rapamycin

MUFA: Monounsaturated fatty acid

NAD: Nicotinamide adenine dinucleotide

NAFLD: Non-alcoholic fatty liver disease

NGS: Next generation sequencing

NIA: National Institute of Aging

NTC: Non-template controls

OO: olive oil

PA: Physical activity

PBS: phosphate buffered saline

PGC1α: Peroxisome proliferator-activated receptor γ co-activator 1 α

PKA: Protein kinase A

PMBCs: Peripheral blood mononuclear cells

PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase

PREDIMED: Prevención con Dieta Mediterránea

PUFA: Polyunsaturated fatty acid

RISC: RNA-induced silencing complex

RNA: Ribonucleic Acid

ROO: Refined olive oil

ROS: Reactive oxygen species

RT-qPCR: Real Time quantitative PCR

SAM: Significance analysis for microarray

SD: Standard deviation

SEM: Standard error of the mean

SFA: saturated fatty acid

SMC: vascular smooth muscle cell

T2DM: Type II diabetes

T3: Triiodothyronine

T4: Thyroxine

TMT: Trail Making Test

UTR: Untranslated region

VLDL: Very low-density lipoprotein

VOHF: Virgin Olive Oil and HDL Functionality

VOO: Virgin olive oil

WNPRC: Wisconsin National Primate Research Center

XN: Xanthohumol

Symbol	Gene / protein name
ABCA1	ATP Binding Cassette Subfamily A Member 1
ABCG2	ATP binding cassette subfamily G member 2
ACSL4	Acyl-CoA Synthetase Long Chain Family Member 4
ADRB2	Adrenoceptor Beta 2
AKAP10	A-Kinase Anchoring Protein 10
AKT	AKT Serine/Threonine Kinase
ALOX5AP	Arachidonate-5-lipoxygenase-activating protein
AP1	Activator protein 1
ARHGAP15	Rho GTPase Activating Protein 15
BECN1	Beclin 1
c-MET	Tyrosine-protein kinase Met
c-MYC	MYC Proto-Oncogene
CD28	Cluster of differentiation 28
CD57	Cluster of differentiation 57
CETP	Cholesteryl ester transfer protein
CHD9	Chromodomain helicase DNA binding protein 9
CNOT6L	CCR4-NOT transcription complex, subunit 6-like
CRY2	Cryptochrome 2
CSNK1A1	Casein Kinase 1 Alpha 1
DAF-16	DAF-16 family binding element
EEF2	Eukaryotic elongation factor 2
eNOS	Endothelial nitric oxide synthase
ERK	Signal-regulated kinase
EXP-5	Exportin-5
FOG2	Zinc Finger Protein FOG Family Member 2
HDAC4	Histone deacetylase 4
HIF1α	Hypoxia inducible factor 1α
IGF1R	Insulin-like growth factor-1 Receptor
IL7R	Interleukin-7 receptor
IRS1	Insulin receptor substrate 1
IRS2	Insulin receptor substrate 2
KIT	Proto-oncogene receptor tyrosine kinase
LDLRAP1	Low Density Lipoprotein Receptor Adaptor Protein 1
LIF	Interleukin 6 Family cytokine
LRP6	Low density lipoprotein receptor Protein 6
MEF2D	Myocyte enhancer factor 2D
MTP	Microsomal transfer protein
MYO1D	Myosin ID
NRF2	Nuclear factor (erythroid-derived 2)-like 2
NUFIP2	FMR1 interacting protein 2
OGT	O-linked-N-acetylglucosamine transferase
P70S6K1	Ribosomal S6 kinase p70
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKFB2	6-phosfofructo-2-kinase / fructose-2,6-biphosphatase 2
POLK	DNA Polymerase Kappa
PPARα	Peroxisome proliferator-activated receptor alpha
PRKAA2	Protein kinase AMP-activated catalytic subunit alpha 2
PRKCB	Protein kinase C, beta subunit
RHeB-1	Ras homolog enriched in brain
SCD	Stearoyl CoA desaturase
SIRPα	Signal-regulatory protein α
SKN1	Protein skinhead-1
STARD7	Star related lipid transfer domain containing 7
SOX11	SRY-Box 11
TNRC6A	Trinucleotide repeat containing 6A
TSC1	TSC complex subunit 1
USH	Zinc finger protein USH
YOD1	YOD1 Deubiquitinase
ZNF148	Zinc finger protein 148

INTRODUCTION

1. Aging situation of European and Spanish population

The demographic trend in Europe indicates a gradual aging of the population. According to Eurostat, average age of European population will increase by 4.2 years between 2014 and 2080. The demographic shift in European countries has been attributed to the progressive decrease in the levels of birth and mortality. The percentage of the population over 65 years old will increase from 18.5% (93.9 million people) in 2014 to 28.7% (149.1 million people) in 2080, assuming an increase of 55.2 million people (1).

If we focus on elderly people (defined as people older than 80 years), the increase in this specific-age group of population will increase from 5.1% in 2014 to 12.3% in 2080, resulting in an increase of more than double respect to the current proportion (from 26 million in 2014 up to 63.9 million in 2080).

Regarding to the European pyramid of population in 2014 and, comparing it with the pyramid predicted for 2080 **(figure 1)**, we can observe that the distribution of the population shifts from a rhomboidal distribution, with a majority percentage of working-age population, to a distribution with the form of an inverted pyramid, equilibrating the working-age population with the retired population. Especially relevant is the increase of centenarians, especially in women.

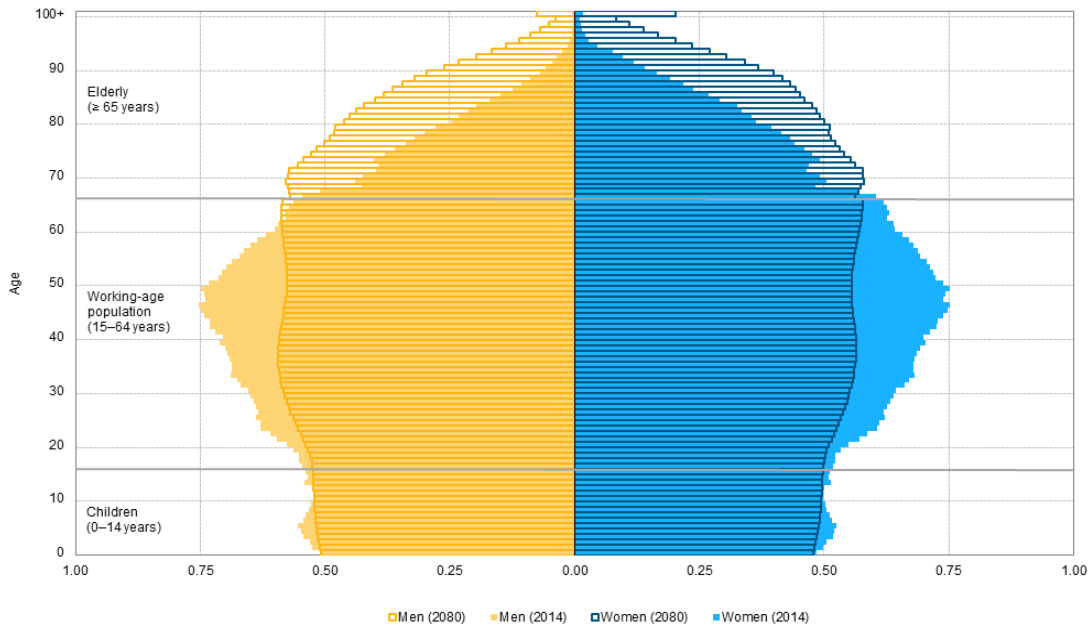

Figure 1: Comparison between the European population pyramid in 2014 with the projected population pyramid for the year 2080 (Source: Eurostat)

If we look at the specific data from Spain, we can see a trend very similar to the demographic shift predicted in Europe, but with a more pronounced aging of the population **(figure 2)**. The causes behind this demographic shift in Spain are the same that in Europe, a lower number of births and deaths. Especially relevant is the increase in lifespan, which will go from 80.2 years in men and 85.71 years in women, to a projection of 88.6 years in men and 91.6 years in women in the year 2060 (2).

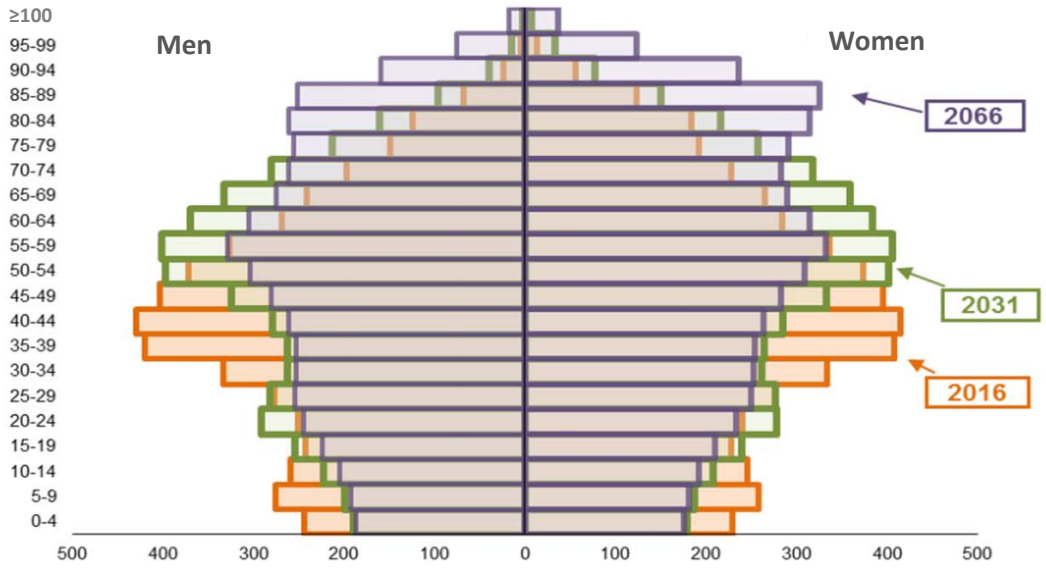

Figure 2: Comparison between the Spanish population in 2016 with the projected population in the year 2031 and 2066 (Source: Spanish national statistical institute, INE)

Not only is Europe facing a gradual aging of its population, but also all the developed or developing countries are facing this circumstance. However, we cannot consider the aging of the population as only a demographic curiosity, but it has been associated to a very significant economic, health and social impact, and can influence the policies that will be carried out in the coming years or decades.

Aging is an irreversible biological process, but it can be modulated. An intervention on aging can not only increase lifespan, but also improve the quality of life, delaying as far as possible the appearance of physiological complications related to age. These actions are framed in the field of prevention, rather than treatment and it is of such importance that more and more countries seek new health policies in order to improve the quality of life of elderly adults. Under this premise, the concept of "healthy aging" describes the health status of people who, even after reaching an advanced age, are free of disease. Unfortunately, the current situation is far from reaching the ideal state of healthy aging. The increase of diseases associated with age is

becoming one of the main health problems in developed and developing countries. Aging is associated with the development of diseases such as Alzheimer's or Parkinson's diseases, Type 2 Diabetes Mellitus (T2D), cancer or cardiovascular disease (CVD) and its associated complications. Definitely, the main objective of healthy aging is not just to live longer; it is to live longer with good quality of life.

In the current situation, we are faced with the following question: What type of intervention is the most appropriate to face aging? There is no clear consensus that can answer this question, although we can find several promising approaches from different areas such as pharmacological, dietary or behavioral intervention (3).

2. Interventions focusing on increasing longevity and promoting healthy aging

As described above, the aging process is modulable. This means that is subjected to environmental factors that influence it. These environmental factors are smoking, pollution, physical activity (PA) and diet (4). In addition to this, we have seen the influence of some drugs on the aging process, as well as on their associated diseases. Some of the interventions that have been studied in aging are pharmacological interventions, interventions on lifestyle and on diet.

2.1 Pharmacological interventions

It has been described that certain drugs can have a potential to enlarge lifespan. Some of the drugs that are thought to have the greatest influence on aging are metformin, rapamycin, statins or aspirin. Metformin, one of the most widely used drug for the treatment of diabetes and prediabetes, is a hepatic activator of AMP-activated protein kinase (AMPK), which, as mammalian target of rapamycin (mTOR), is a sensor of the cellular energy state signaled by the AMP/ATP ratio (ATP, adenosine triphosphate). In worms, rats and mice it has been described that metformin increases lifespan (5), whereas in humans, the treatment with metformin slows the development and progression of diseases associated with aging such as CVD, cancer and neurodegenerative diseases (6, 7). Rapamycin, whose main target is mTOR, has been described to have an effect on cardiac aging, as well as on inflammation, oxidative stress and cellular senescence (8, 9). Furthermore, in mice treated with rapamycin, an increase in lifespan and better general health have been observed, with a greater effect in female mice than in male mice (10). However, the mechanism of action by which rapamycin increases lifespan is not clear because, even though there is an inhibition of mTOR expression, levels of Insulin-like growth factor-1 (IGF-1) decreased, levels of leptin increased and levels of insulin decreased in males and

are softly higher in females, and a worse glucose tolerance has been observed (11). Statins are used to lower blood cholesterol levels, being the drug of choice when analytical values of total cholesterol greater than 200 mg/dl in blood are consistently observed. Apart from the described effect, it has been described that AKT (AKT serine/threonine kinase) activation increases with statins treatment and is associated with lower mortality in elderly people (12). Finally, acetylsalicylic acid, known as aspirin, is used as an anti-inflammatory, analgesic and antipyretic treatment, in addition to its antiplatelet effect. Moreover, it has been described an increased lifespan in crickets and mice treated with this drug (13, 14). **Table 1** shows a summary of pharmacological interventions.

2.2 Interventions on lifestyle

Although lifestyle interventions on aging have not been widely studied, a relationship between both can be observed. For instance, there is an important connection between the feeling of loneliness and healthy aging (15), demonstrating that a lifestyle that fosters social relationships can promote better aging. Within the scope of action on lifestyle, it should be also including the usual practice of PA. The benefits of sports practice on health are well-known (16), but the mechanisms by which PA promotes healthy aging must be studied in depth. PA has been postulated as the only treatment for sarcopenia, which is a disease associated with aging (17). In addition, PA has a protective effect on cognitive deterioration, as well as on neurodegenerative diseases associated with aging such as Parkinson's and Alzheimer's diseases (18). Some studies have shown that, the blood flow velocity in the middle cerebral artery is higher in people who exercise regularly than in sedentary people. This suggest a protective effect with respect to cerebral atrophy associated with aging (19). Another observational study associated an active lifestyle with a lower incidence of neurodegenerative diseases (20). Regarding the length of the telomeres, an important marker of aging, it was observed that, among healthy volunteers, those who were marathon runners had greater telomere length than sedentary volunteers (21). **Table 1** shows a summary of the interventions on lifestyle.

2.3. Nutritional interventions

Diet is one of the most studied environmental factors given tis great impact on aging. In this sense, caloric restriction (CR) has been postulated as one of the most important interventions that allows increasing the expectancy and quality of life of the participants subjected to it (22). There are large number of studies showing that CR can increase lifespan as well as promote an improvement in the quality of life in aging. These studies range from simple animal models such

as *Caenorhabditis elegans* to more complex models such as monkeys (23). However, studies in humans, especially interventional studies, are scarce, mainly due to ethical and social considerations that can limit a sustained CR over time.

2.3.1. Evidences from studies in animal models

CR increases lifespan in different animal models. In 2006, Kaeberlein *et al* observed an increase in lifespan in *Caernohabditis elegans* nematodes, which were deprived of food (24). Other models in which CR has been shown to increase lifespan have been fly *Drosophila melanogaster* (25) and yeasts such as *Saccharomyces cerevisiae* (26).

In the 1980s, using SPF Fischer 344 rats, Yu *et al* observed that 60% of the rats subjected to CR (40% with respect to their *ad libitum* feeding) significantly increased their lifespan comparing to control rats (27). In addition, it was observed that rats under RC had fewer diseases and age-related deterioration (27). In another study, also conducted on SPF Fischer 344 rats, which underwent a smoother CR (8%) along with PA (28), authors found an improvement of muscle composition, oxidative stress, cell death and autophagy (28). Intermittent fasting (IF) is another approach for CR which limits the calories intake certain days while *ad libitum* food intake is allowed in other days (29). In the liver of rats subjected to a CR of 40% and to an IF, that provided access to food every other day, the sensitivity to glucagon was maintained in levels similar to those of young rats in the CR group while the insulin sensitivity was maintained in the IF group (30).

In murine models, mice subjected to an IF with CR of 40% showed a better regulation of glucose homeostasis as well as a 40% reduction in IGF-1 levels. However, these mice exhibited hyperphagia in response to fasting periods (31). In 2007, Varady *et al* presented a study in C57BL/6J mice subjected to different levels of CR with IF (1 day with 50% CR and another day *ad libitum*). The results showed that, although the 50% CR and fasting days modulated the function of adipocytes, no changes in weight or amount of adipose tissue were observed (32).

A meta-analysis that studied CR in rat and mouse models concluded that there is sufficient evidence that CR influences lifespan of mice and lead to a lower rate of age-related diseases incidence. However, more studies are necessary because the effects of CR vary according to the strain of the mouse as well as the design of the intervention based on CR (33).

Regarding more complex animal models such as monkeys, there is greater controversy about the effects of CR on aging. In a study carried out by the Wisconsin National Primate Research Center (WNPRC) on non-human primates (*Rhesus* monkeys) subjected to a CR of 30%, a lower incidence of diseases associated with aging, a smaller loss of brain gray matter, as well as an

increase in lifespan was observed (34). Parallel to this study, the National Institute of Aging (NIA) carried out another study in the same species of monkeys that contradicted the previously described results, since it was not observed that CR increased lifespan (35). However, it has been concluded that there are evidences that CR benefits health in monkeys and that mechanisms explaining these benefits could be similar in humans (34-36). This disparity of results in both studies can be explained by the different experimental designs that were made in both studies (37). However, these results highlight the need to carry out more studies to elucidate the effect that CR has on aging. **Table 1** shows a summary of the evidence in animal models regarding the effect of CR on aging.

2.3.2. Evidences from studies in human populations

2.3.2.1. Effect of CR on markers of aging and cardiovascular health

Regarding the studies carried out in humans, most of them have been observational studies, due to the ethical considerations involved in the development of long-term CR trials. However, it is well described that CR and a healthy dietary pattern have a beneficial effect on longevity and age-related diseases. In the 1950s, certain regions of the world were described in which the number of centenarians was significantly higher. These areas, which were called "Blue Zones", are Sardinia in Italy, Okinawa in Japan, Nicoya in Costa Rica and Ikaria in Greece (38). Interestingly, all these regions are in the same geographical area, they are adjacent to the parallel 42, which suggests that these regions share similar climatological characteristics. It has been proposed the inclusion of a new blue zone that would be located on the island of Menorca given its higher rate of centenarians with respect to the whole Spanish population, but it would be necessary a deeper study of this population to elucidate the causes behind the extreme longevity of this island to consider this area as such (38). The greater longevity in these areas has been related to PA, an active social life, an optimistic attitude and a healthy dietary pattern (39). One of the most studied regions is the Okinawa region. Studying their traditional pattern of diet, it was observed that it has a low animal protein content and is rich in vegetables, fruits and fish, as well as in isoflavones from soy. Additionally, the a caloric content is 17% lower than the Japanese average intake (40). In addition, this dietary pattern is rich in mono and poly-unsaturated fats (MUFA and PUFA, respectively), characteristics shared with other dietary patterns described as healthy, such as the Mediterranean Diet (MD). These patterns have the ability to modulate different molecular processes associated with aging, such as telomere length or oxidative stress (41, 42).

Currently, there are only a few studies in humans that analyze the effect of a CR on aging.

The main CR study conducted in humans is the *Comprehensive Assessment of Long- term Effects of Reducing Intake of Energy* (CALERIE) study, in which healthy women and men with normal weight or overweight were recruited and divided into 4 intervention groups for 6 months: control group, very low calorie diet group (890 Kcal/d) to lose 15% of the weight, group with a CR of 25% from the diet and group with a CR of 25% achieved with a 12.5% CR diet and a 12.5% increase in energy expenditure through PA. The results obtained in this study showed a decrease in weight, body temperature, blood glucose and insulin levels and other the decrease in other biomarkers such as DNA damaged, thyroid hormones T3 (Triiodothyronine) and T4 (Thyroxine) in the groups subjected to CR (43). In the longer term, the two-year CALERIE-2 study did not observe significant differences in body temperature compared to the control group (44). However, these results could be due to a decrease in adherence to the treatment in the CR group, since it was maintained for a long period. Subsequent analyzes of the CALERIE study also showed that CR reduces hepatic lipid levels (45) and improves the cardiovascular risk profile (46). On the other hand, in patients undergoing CR, the levels of ghrelin (considered the hunger hormone) increased, and the levels of cortisol (47) and growth hormone were not modified (48). In addition to these results, a metabolic and behavioral adaptation was observed in patients undergoing CR that resulted in an improved physical capacity and vitality (49), without producing eating disorders (50) in a period ranging from 6 to the 12 months of intervention.

Another study worth mentioning is the *Calorie Restriction with Optimum Nutrition* (CRON) study, which is an observational study of the members of the "Calorie Restriction Society", which voluntarily have undergone a CR of 30% during a period of 15 years. The members of this society had lower levels of LDL (low-density lipoprotein) cholesterol, triglycerides, blood pressure and fasting glucose and insulin, as well as higher levels of HDL (high-density lipoprotein) cholesterol compared with people of the same age and sex who are consumers of a normal western diet (51). Other markers of aging that were modified were body temperature and interleukin levels, which were reduced (52, 53), suggesting that CR can result in an increase in lifespan in humans. Although these results (one observational and one intervention study) are promising, more research is needed in the field of CR in order to confirm its beneficial effects. Both studies are carried out in healthy non-obese of overweight individuals. It would be interesting to describe the effect of CR in a high CVD risk population. A meta-analysis including 42 intervention and observational studies concluded that CR decreases the accumulation of visceral fat, markers of inflammation and leptin levels and increases adiponectin levels (54). In addition, CR also produces a metabolic adaptation of the organism that is expressed as a decrease in energy

requirements, muscle mass and strength, that is more pronounced when CR is maintained long-term (54). **Table 1** shows a summary of the evidence of CR in humans and animal models.

2.3.2.2. Interventions based on IF

Apart from the studies described above based on CR, the IF is another type of nutritional intervention studied with the aim of promoting healthy aging. This type of intervention was studied in the *Alternate Day Fasting in Humans* (FEAST) study, which recruited 8 men and 8 healthy women who underwent fasting every other day for 22 days. After the intervention, the patients lost 2.5% of their basal weight as well as 4% of the fat mass. Although the feeling of hunger increased on the first day and remained high, there was no significant increase in ghrelin levels. Fasting insulin levels increased as well as the oxidation of fatty acids, while fasting glucose levels were not affected (55). These results were confirmed in a subsequent study of the genes involved in the oxidation of fatty acids, which were over-expressed, and the genes involved in glucose homeostasis, which were not affected (56). However, in insulin-resistant individuals, IF may have a beneficial effect on this resistance (57). Another study conducted on young women with overweight or obesity showed that both, CR and IF, reduced weight, leptin, total cholesterol and LDL cholesterol levels, while increased insulin sensitivity (58). However, some disadvantages associated with IF such as bad mood or increased visceral fat due to inactivity associated with fasting days have been described (59). In summary, both, sustained CR and IF, can help increase lifespan as well as healthy aging, as studies in both animal and human models suggest. **Table 1** shows a summary of the evidence of IF in animal models and in humans.

2.3.2.3. Selective nutrient restriction

Finally, it is important to highlight some studies that propose only restricting some nutrients as an alternative to complete CR. Although it has been observed that diet has a very important effect on increasing lifespan, it is not known what type of dietary intervention is the one that produces a greater, safer and more sustained effect. In this regard, diets with protein restriction have been shown to be more effective in the repression of mTOR compared with diets with CR in mice (60). In this sense, a restriction of serine, threonine and valine in yeasts produces a resistance to stress, as well as an increase in longevity (61). However, a study carried out in *Drosophila melanoganster* showed that a CR produced a greater increase in lifespan compared to a carbohydrate restriction, independently of caloric intake (62). In rats, a specific restriction of tryptophan has been related to an increase in longevity (63, 64), as well as a restriction in the intake of L-methionine from 0.86% to 0.17% increased lifespan 30% in Fisher 344 rats (65). Diets low in methionine also decrease levels of IGF-1, glucose and insulin and confer greater resistance

to liver damage promoted by oxidative stress (66). Although some studies suggest that general control nonderepressible 2 (GCN2) protein, an aminoacid sensor, and mTOR play a role in mediating the beneficial effects of restricting some amino acids (67) it is not yet clear which molecular mechanisms are involved.

In a murine model of breast and prostate cancer, it was observed that a protein restriction decreased tumor growth and mTOR activity in the tumor, also leading to lower levels of serum IGF-1 (68). In mice, diets low in protein and high in carbohydrates also produce a decrease in mTOR levels and an increase in lifespan (60). In humans, serum IGF-1 levels are not affected by CR, but they decrease with a low protein diet (69). **Table 1** shows a summary of the evidence in animal models regarding the specific restriction of nutrients.

Intervention type	Intervention				Effect	References
Pharmacological interventions	Metformin → AMPK				↑ Lifespan ↓ CVD, ↓Neurodegenerative diseases, ↓ Cancer	(5-7)
	Rapamycin → mTOR				↓ Inflammation, ↓ Oxidative stress, ↓ Cellular senescence, ↓ Cardiac aging	(8-11)
	Statins → AKT				↓ Old mortality	(12)
	Aspirin → AMPK				↑ Lifespan, ↓ Inflammation	(13, 14)
Lifestyle interventions	Social				↑ Lifespan	(15)
	Physical activity				↑ Telomeres length ↓ Sarcopenia, ↓ Neurodegenerative diseases, ↓ Cerebral atrophy	(17-21)
Nutritional interventions	Caloric restriction	Animal models	*C. elegans*		↑ Lifespan	(24)
			Flies		↑ Lifespan	(25)
			S. cerevisiae		↑ Lifespan	(26)
			Rats		↑ Lifespan, ↓ Age-related diseases	(27, 28, 30)
			Mice		↑ Lifespan, ↓ Age-related diseases, Glucose homeostasis	(31-33)
			Monkeys	NIA study	= Lifespan	(35-37)
				WNPRC study	↑ Lifespan, ↓ Age-related diseases, ↓ Cerebral grey mass loss	(34, 37)
		Humans	CALERIE study		↓ Body temperature, ↓ Glucose and insulin levels, ↓Thyroid hormones, ↓Hepatic lipids, ↓ Cardiovascular risk, ↑ Ghrelin, metabolic adaptation	(43-46, 48-50)
			CRON study		↓LDL, ↓ Triglycerides, ↓Blood pressure, ↓Glucose, ↓ Insulin, ↓ Body temperature, ↓ Interleukins, ↑HDL, metabolic adaptation	(51-53)
	Intermittent fasting	Humans	FEAST study		↓Weight, ↑ Insulin, ↑Fatty acid oxidation, ↑Hunger feeling	(55-57)
			Premenopausal women		↓ Weight, ↓LDL, ↓ cholesterol, ↓leptin, ↑Insulin sensitivity	(58, 59)
	Selective restriction of nutrients	Animal models	S. cerevisiae	Serine, threonine and valine restriction	↑ Lifespan, ↑ Stress resistance	(61)
			D. melanogaster	Carbohydrates restriction	↑ Lifespan	(62)
			Rats	Tryptophan restriction	↑ Lifespan	(63, 64)
				L-methionine restriction	↑ Lifespan, ↓ Insulin, ↓Glucose, ↓IGF-1, ↑Hepatic damage resistance	(65, 66)
			Mice	Proteins restriction	↓ Tumoral growth ↓ mTOR activity, ↓IGF-1	(60)
				Carbohydrates restriction	↓ mTOR activity, ↑ Lifespan	(60, 69)

Table 1: Summary of all type of interventions described with an effect in healthy aging and longevity

3. The Mediterranean Diet: a great allied in healthy aging

3.1 MD: a healthy diet pattern

MD is defined as the traditional diet of the countries around Mediterranean Sea. This diet is characterized by a high consumption of olive oil (being the main source of fat), vegetables, legumes, fruits and nuts, with a moderate consumption of blue fish and white meat and with a low consumption of other types of fat and red meat **(figure 3)**. In addition, MD is associated with a moderate consumption of alcohol, usually from fermented alcoholic beverages such as wine or beer and with moderate PA (70). Among the beneficial properties associated to MD, it is worth highlighting the improvement in cognitive function, which indicates an important effect on the quality of life in elderly people (70). The importance of MD in maintaining an optimal state of health is well demonstrated. The first evidence about the benefits of MD were described in 1958 by Ancel Keys in the *7 countries study*, tat describe a lower prevalence of CVD in countries around Mediterranean Sea that shared the same dietary pattern (71) . The *Prevención con Dieta Mediterránea* (PREDIMED) study has demonstrated its clear cardioprotective profile with respect to the low-fat diet recommended by the American Heart Association (72). Some of the potential mechanisms of this effect are related to factors such as inflammation, oxidation and lipid profile, among others (72) . It has been described that, in these Mediterranean countries, the prevalence of cardiovascular disease is lower (42), a greater longevity has also been observed in populations with a high adherence to the MD (73, 74). These results could indicate that the combination of a CR together with the beneficial properties of MD could have a very positive effect on healthy aging.

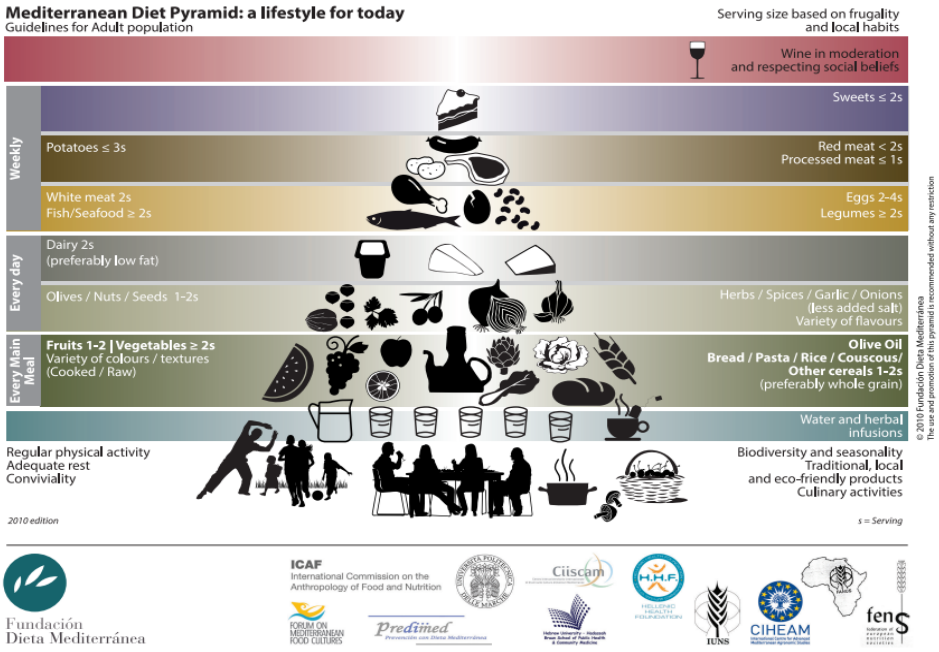

Figure 3: Food pyramid of the Mediterranean Diet (Source: Mediterranean Diet Foundation)

Although we are beginning to discern the mechanisms that could explain the effect of MD on aging, such as the length of telomeres (41), the effect of this diet on other markers of aging has not been fully deciphered.

3.2. The role of olive oil in health

Olive oil is one of the most representative foods of MD and to which unique health properties have been widely described (75-77). The formulation of olive oil can vary greatly depending on the type of olive used for the extraction, the variety of the climate, the soil and the extraction procedure. According to the extraction procedure of olive oil, during the process, it can be obtained extra virgin olive oil (EVOO), virgin olive oil (VOO), olive oil (OO) and refined olive oil (ROO) being the highest quality the EVOO (78).

The composition of olive oil in fatty acids is oleic acid (55-85%), palmitic acid (6-20%), linoleic acid (2-20%), stearic acid (0.3-5%), palmitoleic acid (0.3-2%) and linolenic acid (0.1-1.5%) (79). Olive oil also has a large number of polyphenols that contribute to its healthful properties (80).

The use of olive oil as a cardio protective agent has been also studied. The observational study "Study of Three Cities" shows a relationship between the consumption of olive oil and a lower prevalence of strokes in elderly adults (76). In addition to this, PREDIMED study shows a lower percentage of stroke in high cardiovascular risk volunteers under a MD supplemented with olive oil compared with the control group that consumed a low-fat diet (72).

Oleic acid, the main fatty acid in olive oil, increases HDL cholesterol levels and decreases the oxidation of LDL cholesterol in humans (75, 81). Additionally, polyphenols present in olive oil have been shown to have a chemo-protective effect. The most important polyphenol is hydroxytyrosol, which is a phenolic alcohol involved in the elimination of oxygen free radicals species (ROS) and with an antioxidant effect (82). Moreover, hydroxytyrosol has also been shown to have an anti-inflammatory, antiplatelet, antiatherogenic (83), antitumor (84) or neuroprotective effect (85, 86). Apart from hydroxytyrosol, other polyphenols present in olive oil such as secoiridoids, hydroxy-isochromans, flavonoids and lignans have an effect on microbial activity, inflammation, oxidation, platelet aggregation and on the lipoprotein profile (87). However, this healthy effect of olive oil could be modified according to the amount of polyphenols present in it, an amount that is affected by environmental factors, type of tree and olive variety (88).

Despite these results, the effect of olive oil intake at the molecular level has not been described in depth. In this regard, it is unknown how it modulates gene expression in humans, even though this modulation has been seen in cell lines and in murine models (89). In the *Virgin Olive Oil and*

HDL Functionality (VOHF) study, the effect of different types of virgin olive oils (some of them enriched in polyphenols) on the modification of HDL as well as on other parameters associated with cardiovascular benefits was analyzed in 33 hypercholesterolemic participants. These patients took three different types of oil enriched in its own polyphenols and authors observed that the intake of EVOO and its enriched variants modified the functionality of HDL cholesterol, as well as the expression of proteins related to cholesterol metabolism, response to oxidation, coagulation , lipid transport or immune system (81). Other postprandial study carried out with 6 healthy volunteers (90) and a 3 months study with 90 healthy volunteers (91) did a molecular approach analyzing the changes in the expression levels of genes involved in inflammation, oxidative stress, lipid metabolism, cellular apoptosis or DNA damage after an intake of EVOO, showing a downregulation of proatherogenic genes or upregulation of anti-inflammatory genes producing a cardioprotective effect (90, 91).

3.3. The role of beer in health

Beer is one of the most popular fermented beverages in Europe and is original of the Mediterranean area. Fermented drinks are those in which, during the manufacturing process, the food source is modified by yeasts and, as a consequence, some changes in the nutritional composition happen (92). This fermentation process is considered one of the oldest biotechnological applications in food. Moreover, appearance of beer in the human diet has been dated around 5000 BC. In 2016, 39,000 million liters of beer were produced in the European Union, with 900 million liters corresponding to the production of beer without alcohol (considered beer with 2% or less of alcohol). Beer is mainly composed by water, hops, yeast and malted cereals (usually barley but can be made from other cereals). In the brewing process, the grain is introduced into water where the grain germinates and enzymes break the cell wall and starch producing the softening of the cereal. Once the optimum point of germination is reached, the cooking process starts, which will stop the germination and eliminate part of the humidity of the grain. In this process organoleptic changes occur in the color and flavor of the grain due to the high temperature, turning the grain into malt, which is stored for several weeks. Subsequently, the malt is grinded and soaked with hot water to gelatinize it and to facilitate the digestion process carried out by amylases. The liquid produced is collected and boiled with the hops. Later, a clarification and cooling process takes place adding the yeast strain specific of the type of beer that is being manufactured. In the fermentation process, yeasts convert the sugars into ethanol and CO_2. In addition, other organoleptic changes that give the final taste to beer

also occur. Once the beer fermentation process is complete and the yeasts are removed, the beer is stabilized, clarified and prepared for consumption (93).

The nutritional composition of beer consists in carbohydrates, amino acids, vitamins (mainly from group B) and minerals such as selenium, potassium, zinc or magnesium **(table 2)**. The nutritional composition of beer has been related to its healthy properties (94). It has been observed that beer consumption has an effect on factors related to cardiovascular risk such as higher levels of HDL cholesterol and lower levels of LDL cholesterol oxidation, as well as anti-inflammatory properties (95, 96).

Table 2: Beer composition per 100 g (Source: Spanish Food Composition Database)

Beer composition per 100 g					
Beer			Non-alcoholic beer		
Component	Value	Units	Component	Value	Units
Energy	176 (42)	kJ (kcal)	Energy	108 (26)	kJ (kcal)
Alcohol (ethanol)	3.96	g	Alcohol (ethanol)	0.33	g
Carbohiydrates	3.12	g	Carbohiydrates	5.4	g
Fat	0	g	Fat	0	g
Protein	0.5	g	Protein	0.375	g
Water	92.4	g	Water	93.9	g
Vitamin A	0	ug	Vitamin A	0	ug
Vitamin D	0	ug	Vitamin D	0	ug
Vitamin E	0	mg	Vitamin E	0	mg
Folate	6.3	ug	Folate	15	ug
Niacine	0.43	mg	Niacine	0.7	mg
Riboflavine	0.033	mg	Riboflavine	0.0055	mg
Tiamin	0.003	mg	Tiamin	0.006	mg
Vitamin B12	0.15	ug	Vitamin B12	0.1	ug
Vitamin B6	0.062	mg	Vitamin B6	0.034	mg
Vitamin C	0	mg	Vitamin C	0	mg
Calcium	8	mg	Calcium	5	mg
Iron	0.01	mg	Iron	0.02	mg
Potasium	37	mg	Potasium	40	mg
Magnesium	9.6	mg	Magnesium	7.7	mg
Sodium	4.4	mg	Sodium	2.6	mg
Phosforus	55	mg	Phosforus	20	mg
Iodine	8	ug	Iodine	1	ug
Selenium	1.2	ug	Selenium	1.2	ug
Zinc	0.006	mg	Zinc	0.008	mg

In addition to these effects, other potentially antioxidant, anti-carcinogenic, anti-inflammatory, estrogenic and antiviral effects of beer have been described, mainly in *in vitro* models, and have been based on specific compounds of beer, but not on the whole drink (97-99). Many of these compounds are polyphenols, which come mainly from the malt (approximately 70-80%), while the remaining 20-30% comes from hops (100). Up to 47 different polyphenols have been described in beer, including simple phenolic acids, flavonols, flavones, prenylvanoxanes, hydroxycinnamoylquinic acids, alkylmethoxyfenols, alpha- and iso-alpha-acids (101). Among all polyphenols in beer, it is important to highlight 8-prenylnarigerin (8-PG) and isoxanthohumol

(IX) (101). 8-PG has a beneficial effect in protecting against metabolic dysfunctions associated with T2D in mice (102), anticancer activity in U-118 MG cells (103) or activation of PI3K, AKT, and *p70s6k1* (ribosomal S6 kinase p70) in myotubes, leading to a better recovery from muscular dystrophy (104). Studies in mouse models have elucidated the molecular mechanisms associated with the effects of xanthohumol (XN), the precursor of IX. Thus, it has been shown that XN reduces the growth of the prostate tumor (105), reduces the accumulated cholesterol in the aortic arch and increases HDL-cholesterol, an effect that is mediated by a reduction in the expression of cholesteryl ester transfer protein (*CETP*) (106), and improves the atherogenic profile of mice deficient in apolipoprotein E (*APOE*) (107). In Zucker rats treated with XN, a reduction in oxidized products derived from the oxidation of fatty acids and ROS species was observed, mediated by an improvement of mitochondrial activity attributable to XN (108).Beer consumption also prevented the mitochondrial stress induced by Adriamycin in heart and liver mitochondria in a rat model treated with beer (109). In animal models closer to humans, such as pigs, it was observed that the consumption of beer improved the prognosis in post-infarction cardiac recovery, in addition to having other cardioprotective effects such as the greater antioxidant capacity of HDL, the activation of the AKT/eNOS (endothelial nitric oxide synthase) pathway or the greater resistance to oxidation of LDL in the coronary arteries (96, 110).

3. Nutrient sensor pathways

In order to carry out an effective intervention on aging, the first step that must be taken is the understanding of how the aging process occurs and what type of factors are associated to aging. Some of the processes involved in aging are: genetics factors (variants in genes involved in aging pathways and genomic instability), telomeres´ shortening, epigenetic alterations, loss of protein homeostasis, alteration of intercellular communication pathways , loss of regenerative capacity of stem cell tissues, cellular senescence processes, mitochondrial dysfunction and loss of regulation of sensory pathways of metabolic state (111).

One of the most basic function of living organisms is nutrition, function that must respond, from a physiological point of view, to the fluctuation in nutrient levels. The scarcity or abundance of nutrients such as sugars, lipids, amino acids, vitamins, minerals, etc. is detected by multiple sensory pathways that trigger responses whose main function is to maintain the stability of these nutrients, the so-called "nutrient homeostasis". In a situation of abundance of nutrients, the activated pathways are anabolic and storage pathways, while in a situation of scarcity, catabolic pathways and mobilization of previously stored reserves will be activated (112). Less sensitivity to nutrient levels, as well as the deregulation of these pathways has been associated

with the appearance of diseases such as cancer, neurodegenerative diseases or metabolic syndrome and aging (113, 114).

Some of the main nutrient sensing pathways that are affected during the aging process are: the IGF1/PI3K/AKT/FOXO/mTOR (PI3K, phosphatidylinositol-4,5-biphosphate 3 kinase; FOXO, forkhead box O) signaling pathway, whose over-expression is related to aging; and the AMPK/SIRT1/PGC1α (SIRT1, Sirtuin 1; PGC1α, peroxisome proliferator-activated receptor γ co-activator 1 α) signaling pathway, which under a CR is over-expressed and may have a protective effect against aging (111, 115).

4.1. Description of the IGF-1/PI3K/AKT/FOXO/mTOR and AMPK/SIRT1/PGC1α pathways and their role in metabolic and cardiovascular diseases

The primary regulation of IGF-1/PI3K/AKT/FOXO/mTOR pathway is mediated by fluctuations in glucose levels. High blood glucose levels promote pancreatic insulin release that leads to an increase in IGF-1 levels. IGF-1 binds to its membrane receptor (IGF-1R) leading to its autophosphorylation and simultaneously activating PI3K. The activation of PI3K (in its p85 unit) gives rise to a conformational change that leads to the binding of the p110 catalytic subunit. Activated PI3K phosphorylates PIP2 (phosphatidylinositol-4,5-biphosphate) by adding a new phosphate group and transforming it into PIP3 (phosphatidylinositol-3,4,5-triphosphate), which activates AKT.

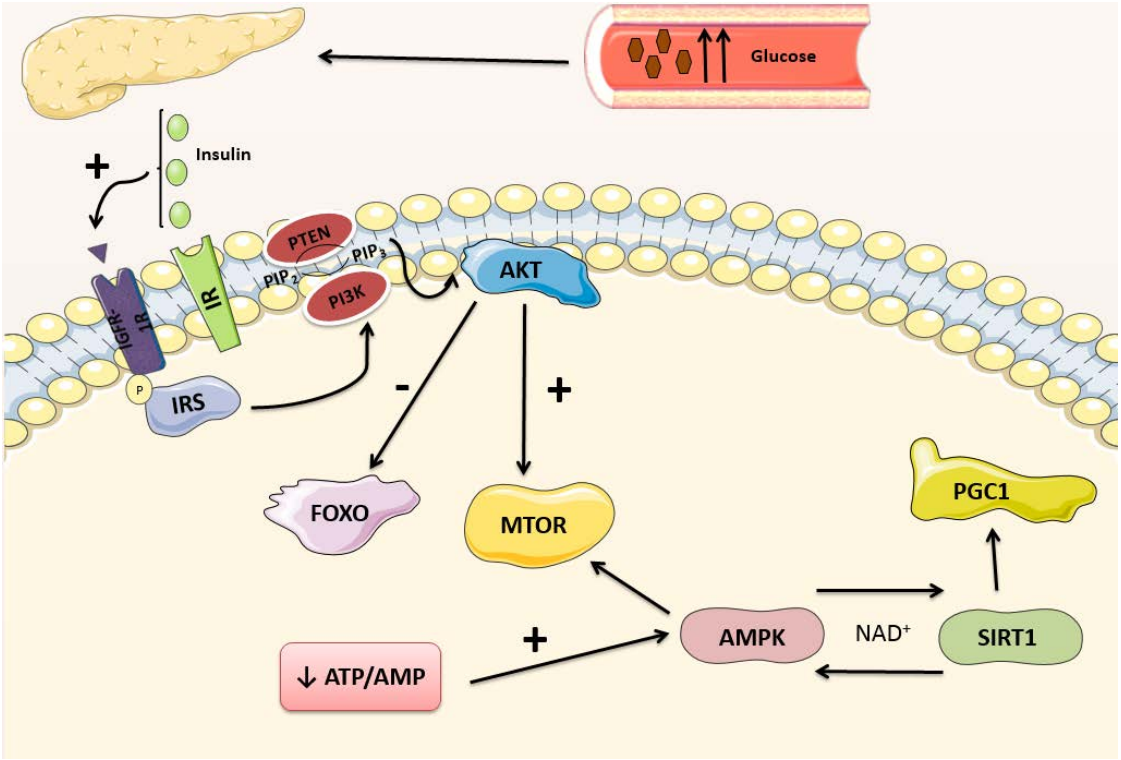

Figure 4: Regulation and effect of nutrient sensing pathways IGF-1/PI3K/AKT/FOXO and AMPK/SIRT1/mTOR pathways

This activation produces, in turn, the activation of mTOR and the inhibition of FOXO **(figure 4**). FOXO is involved in the regulation of genes related to metabolism, apoptosis and cell cycle. This route is highly regulated by diet and it is modulated by CR (116).

There is great scientific evidences supporting the notion that the reduction of mTOR expression is related to an increase in lifespan in animal models such as yeast, worms, fly and mouse (117). mTOR is a protein kinase composed of 2 complexes mTORC 1 and mTORC 2 (113). Instead of both complexes has different functions, both are involved in the pathogenesis of diabetes, as well as in the metabolism of pancreatic β-cells (113). mTORC 1 and mTORC 2 activate transcription factors such as HIF1α (hypoxia inducible factor 1α) and MYC protooncogene (c-Myc), which induce the synthesis of glycolytic enzymes (118, 119). HIF1α is related to the development of T2D (120) and the overexpression of c-Myc in pancreatic β cells reduces the expression of the insulin gene, promoting the development of diabetes (121). FOXO is a key transcription factor in a multitude of processes and is well conserved (122). Among the most relevant functions are its antioxidant activity, stress response, autophagy, apoptosis or cell proliferation (122).

AMPK/SIRT1/PGC1α signaling pathway is an important regulator of energy status. High levels of AMP (adenosine monophosphate) indicates low cellular energy and the necessity of the activation of catabolic routes to increase energy availability. High levels of AMP or, more specifically, a low ATP/AMP ratio activates AMPK **(figure 4)**, which regulates molecules such as Sirtuins, histone deacetylase enzymes that are regulated at the epigenetic level and which have been shown to play an important role in the regulation of nutritional status, as well as in longevity (123, 124). SIRT1 is a sensor of NAD^+ (nicotinamide adenine dinucleotide) levels, key for the regulation of energy metabolism(125). With low levels of AMP, sirtuins levels increase. Among the activated SIRT-1, is PGC1α which stimulates the fatty acids oxidation and mitochondrial biogenesis (123). In turn, AMPK inactivates mTORC1 (111), which implies an interrelation between both pathways of nutrient sensors with an antagonistic effect on the aging process.

4.2. Relationship of nutrient sensing pathways with aging

The regulation of nutrient sensing pathways plays a crucial role in the aging process. As explained in **figure 5**, the IGF-1/PI3K/AKT/FOXO/mTOR pathway regulates the expression of mTOR, whose over-expression is related to aging. On the other hand, signaling of AMPK/SIRT1/PGC1α, which could be regulated by CR, is also related to aging. In *Caenorhabditis elegans*, lower signaling of the insulin/IGF-1 cascade leads to a greater response to stress and

longevity mediated by repression of the FOXO DAF-16 family binding element (*DAF-16*) protein and an accumulation of *skn1* (protein skinhead-1) in the nucleus (126). Moreover, in this animal model, a trial with IF in a two-day food/fasting cycle confirmed the role of *DAF-16* in the increase in longevity (127). In this study, AP-1 (activator protein 1) was also identified as a modulator of longevity mediated by IF (127). In *Drophosila melanoganster,* it has been observed that dFOXO, analogous to FOXO3 in mammals, contributes to the modulation of the effect of CR, although it is not essential for the increase of longevity (128). In a murine model, it has been suggested that the increase in longevity due to CR can be mediated by FOXO3, which has an influence on autophagy and oxidative stress (129, 130). CR in rats and humans results in transcriptional and post-transcriptional modifications in genes belonging to the PI3K/AKT/FOXO signaling cascade(131). Moreover, the study of polymorphisms in these genes has allowed the association of the presence of certain polymorphisms with higher lifespan (131) as well as a lower prevalence of CVD (132).

CR increases the metabolic activity of pathways such as glycolysis, gluconeogenesis, oxidative phosphorylation or mitochondrial function, while producing a repression of the insulin/IGF-1 pathway in skeletal muscle in humans and rats (133) **(figure 5)**. In *Drophosila melanoganster* subjected to a CR, mTOR activity decreased, producing an increase in longevity (134). In *Caenorhabditis elegans,* it was observed that the raptor mutation, equivalent to mTOR or mTORC1, can increase the lifespan (135). In this species, it was also observed that IF could increase the lifespan throughout the modulation of the expression of *rheb-1* (Ras homolog enriched in brain), which activates mTORC1 (136). However, the mechanisms involved in the increase in lifespan in worms are not yet clear because other studies carried out by Kaeberlein *et al* and Lee *et al* have observed that the increase in lifespan is independent of the insulin/IGF1 and Sirt2 (Sirtuin 2) signaling cascade (24, 137).

In addition to this, studies on polymorphisms in the gene encoding the IGF-1 receptor have shown that higher levels of IGF-1 in plasma and lower activity of the IGF-1 receptor have been associated with extreme longevity (138). Interestingly, in another study conducted by Milman *et al* it was shown that lower levels of plasma IGF-1 were associated with greater survival in nonagenarian women while the effect in men was not observed (139).

Using samples from studies conducted in humans, it was observed that the addition of serum from patients of the FEAST study to HEPG2 cells resulted in a reduction in its proliferation and in an increase in the expression of SIRT1 (140). The increase in SIRT1 has also been observed in cells with serum from patients undergoing CR of the CALERIE study (140) as well as in muscle samples from patients in the FEAST study (56). Studies in yeasts point to the crucial role of the synthesis of NAD^+ and SIRT2 in the increase of lifespan (141). In mice with Cockayne syndrome,

a disease characterized by accelerated aging, it was observed that one of the causes of accelerated aging is the deterioration in the DNA repair system that leads to a decrease in the activity of SIRT1 (142). In addition, an increase in SIRT1 has been observed in mice subjected to CR in certain regions of the brain (143). In addition, an increase in the expression of SIRT1 in the brain is related to an increase in life expectancy (144).

Although the relationship between SIRT1 and the increase in lifespan is well described, the mechanism of action has not yet been correctly described. However, it has been observed that SIRT1 increases the expression of genes related to neuronal signaling and that an overexpression of SIRT1 improves neuronal activation as well as the neuronal adaptive response to CR (143, 144).

All these studies demonstrate the importance of the regulation of SIRT1 to promote healthy aging. For this reason, the use of synthetic activators of SIRT1 has been proposed to increase life expectancy as well as healthy aging. However, since the molecular mechanisms by which SIRT1 affects longevity have not been clearly described, the effectiveness of these treatments can not be affirmed (145). Some compounds naturally present in foods such as resveratrol activate SIRT1 *in vitro* (146) **(Figure 5).**

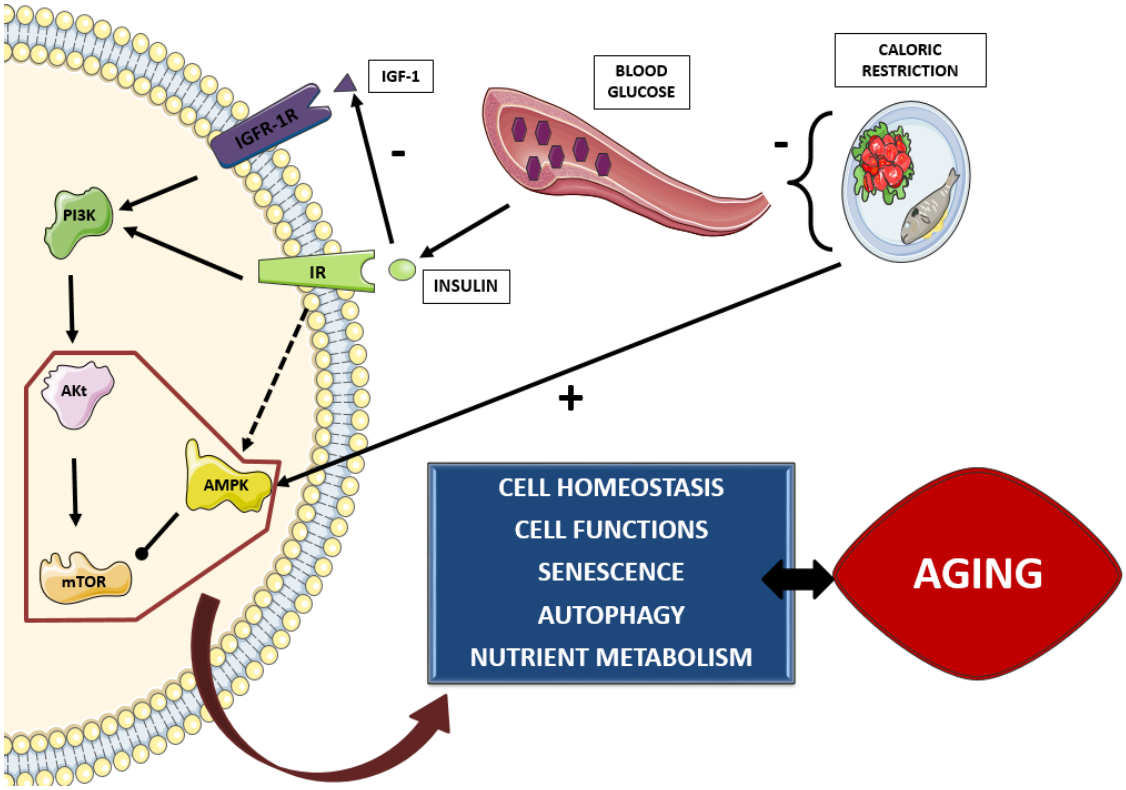

Figure 5: Relationship between caloric restriction and aging from nutrient sensory pathways. Modification of Micó V. et al. (2017) (publication annex)

5. MicroRNAs

5.1. Synthesis and function of microRNAs

MicroRNAs are non-coding RNA sequences with an approximate length of 22-24 nucleotides located in inter- or intragenic regions. Sometimes the microRNAs could be concentrated in certain regions of the RNA that constitute policistronic regions; being very common the transcription of theses microRNAs simultaneously (147). MicroRNAs could be synthesized through two different pathways: the canonical pathway and the non-canonical pathway, being the canonical pathway the most common form of synthesis in animals (148, 149).

In the canonical pathway **(figure 6a)**, RNA polymerase II transcribes the microRNA inside the nucleus, generating a precursor called pri-microRNA (primary) with a hairpin-shaped structure (150). The pri-microRNA goes through different maturation processes before moving on to the next phase of its synthesis (151). This process begins with a microprocessor complex of the pri-microRNA that is responsible for cleaving the pri-microRNA to produce a shorter hairpin molecule of approximately 65 nucleotides called pre-microRNA (precursor) (152). The microprocessor complex of the pri-microRNA is composed by RNA III Drosha and DGCR8. RNA III Drosha is an nuclear endonuclease of 160 kDa approximately. DGCR8 is a protein of approximately 90 kDa that is found in the nucleus and nucleoplasm. This protein recognizes the pri-microRNA and positions the Drosha catalytic sites for the processing of the pri-microRNA (153).

Pre-microRNA is exported to the cytoplasm via Exportin-5 (*EXP-5*) (154). This protein forms a complex together with the nuclear protein RAN-GTP (GTP, guanosine triphosphate) (154). This protein is found in the nuclear membrane. Upon binding, GTP is hydrolyzed opening a pore from which the pre-microRNA exits to the cytoplasm (148, 151, 155).

Once it is in the cytoplasm, the pre-microRNA is subsequently cleaved by Dicer. Dicer is an approximately 200 kDa RNase type III endonuclease (156). Dicer forms a catalytic center that processes the pre-microRNA to produce a double-strand sequence of approximately 22 nucleotides (155). After this processing, both chains of the mature microRNA (3p and 5p) are associated with Argonaute proteins (AGO) forming an RNA-induced silencing complex (RISC) (148). This complex guides the mature microRNAs towards their target gene, where the microRNA produces translational repression and mRNA degradation (148).

The other pathway for the synthesis of microRNAs is the non-canonical pathway **(figure 6b)** that originates from sequences found in introns or in genes that encode proteins. These microRNAs are called mirtrons and they are processed directly from the gene they belong to without the intervention of Drosha or DGCR8. Through the excision of the gene to which the microRNA belongs, an intermediate precursor is generated and processed by the lariat debranching enzyme obtaining a pre-microRNA that is exported to the cytoplasm via *EXP-5* and enters the canonical route (157). Recently, a third synthesis pathway called "simtrom pathway" (splicing-independent mirtron-line microRNAs) has been described. In this pathway, the mirtrons are processed by Drosha and by another yet unknown protein similar to DGCR8 (157). Non-canonical pathways produce mature microRNAs with the same gene silencing functions as canonical microRNAs.

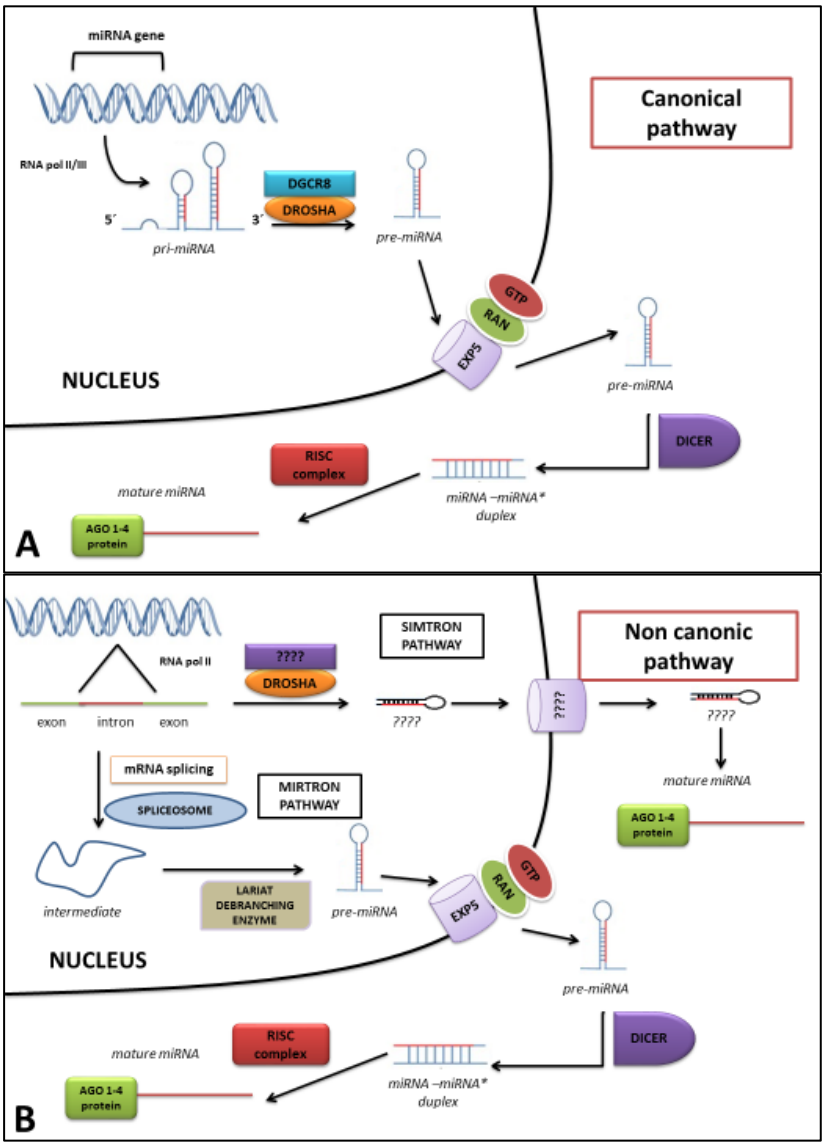

Figure 6: Description of microRNAs synthesis. A) Description of canonical pathway and b) description non-canonical pathway

As described above, the main function of the microRNAs is to repress the expression of their target genes by binding specifically to them through base complementarity. However, the complementarity between microRNA and target mRNA is not always perfect. Depending on the degree of complementarity, the microRNAs can induce the degradation of their target mRNA (total or high complementarity), or their deadenylation or transductional repression (partial complementarity). The binding between the microRNA and the target mRNA sequence occur between the recognition region of the 3'UTR (untranslated region) of the mRNA and the 5' sequence of the microRNA called "seed sequence" (149, 158). However, microRNA binding to 5'UTR and coding sequences have also been described (159)

5.2. Relationship between microRNAs and metabolism and metabolic diseases. Modulation mediated by diet.

Considering the large number of microRNAs discovered in humans, it is believed that more than 60% of human genes have binding sites for microRNAs (160). The microRNAs have a pivotal role in the regulation of several biological processes as well as in the development of disorders and metabolic diseases. Some of the processes that have been shown to be influenced by microRNAs are the metabolism of glucose, lipids or amino acids and some of the diseases that have been associated to microRNAs are diabetes, obesity, CVD, etc.(161-164).

The onset and progression of age-related diseases such as T2D, CVD, inflammation or cancer are modulated by microRNAs (124, 161, 165, 166). Regarding to T2D, it is known that the let-7 family modulates glucose homeostasis and insulin sensitivity in mice (167, 168). Other microRNAs that regulate the insulin signaling pathway are miR-33, miR-103, miR-107 and miR-29 (124, 169-172). In lipid metabolism, it has been shown that several microRNAs could regulate lipoproteins. For example, in mice and non-humans primates, antagonism of miR-122 reduces plasma levels of LDL and VLDL (very-low density lipoprotein) (173, 174)and miR-142, miR-181 and miR-383 were overexpressed in mice fed a high-fat diet (175, 176). In the case of miR-30c is an important regulator of VLDL metabolism because of it targets the microsomal transfer protein (*MTP*) that regulated VLDL production (177). In addition to this, miR-30c and miR-192 overexpression have an effect on the expression of genes related to lipid metabolism (178). Moreover, the expression of these microRNAs could be regulated by docosahexaenoic acid (DHA). Regarding to this, miR-107, a microRNA related to circadian rhythm could be also regulated by DHA in CACO2 cell line model (179). In wistar rats, a high-fat diet reduces the expression of miR-145 (180). The regulation of HDL by miR-33a/b has been deeply studied, being the most relevant microRNA in its regulation of HDL and reverse cholesterol transport (165, 171). Other microRNAs that

regulated HDL are miR-128-1 and miR-148a, whose overexpression results in lower levels of circulating HDL and *ABCA1* (ATP binding cassette subfamily A member 1) expression (172). In a miR-223 knock out (KO) mice model, HDL levels are higher in miR-223 KO mice than in control mice (181). In atherosclerosis, a disorder preceding the development of CVD, the absence of miR-21 in macrophages results in accelerated atherosclerosis, plaque necrosis, and vascular inflammation (182). In healthy women who consumed a diet rich PUFA for 8 weeks, the levels of circulating miR-328, miR-330-3p, miR-221 and miR-125a-5p were reduced while miR-192, miR-486-5p, miR-19b, miR-106a, miR-769-5p, miR-130b and miR-18a were overexpressed (183). Other volunteers who consumed a high-protein diet showed decreased levels of HDL-associated miR-223 (184).

Finally, aging is related to a low-grade level of chronic inflammation. Some microRNAs that have an important function as regulators of inflammation are miR-146, miR-155 and miR-21 (185). **Table 3** shows a summary of the relationship of microRNAs and the pathways they modulate with their relationship with the diet.

5.3. Circulating microRNAs: biomarkers of health status and response to treatment.

In addition to their regulatory role in a great number of cellular processes, microRNAs have been detected in plasma and other biofluids (186). MicroRNAs circulate in the bloodstream associated with exosomes, lipoproteins or protein complexes that protect them from possible degradation mediated by RNAses (186). In this sense, the role of circulating microRNAs as biomarkers of different diseases and also of nutritional status has been postulated (187).

Circulating miR-1, miR-208a, miR-208b and miR-133a are overexpressed within 2 hours after an acute myocardial infarction (AMI) (188, 189) and circulating miR-423-5p increases its expression in heart failure (190, 191).Circulating levels of miR-9 and miR-126 are lower in patients with hypertension in comparison with healthy individuals (192). On the other hand, the levels of miR-221 and miR-222 were significantly lower in atherosclerotic patients compared to healthy patients (193). Circulating miR-192, a microRNA related to inflammation, has been found overexpressed in patients with chronic inflammation with respect to healthy patients (194). In addition, circulating levels of miR-30c can also regulate macrophage-mediated inflammation and atherosclerotic pathways (195). Finally, a large number of microRNAs are associated with the initiation and progression of cancer, suggesting the possible future importance of the measurement of circulating microRNAs as biomarkers of cancer prognosis and treatment (196). miR-21-5p, miR-20a-5p and miR-223-3p have been found to be overexpressed in lung cancer in stages I and II (197).

Not only have changes in circulating microRNAs been observed associated with disease or nutritional status, circulating microRNAs levels could change after performing PA. Circulating levels of miR-208b and miR-221 were higher after 3 months of intense basketball training (198). Moreover, a battery of 12 microRNAs related to inflammation (let-7d-3p, let-7f-2-3p , miR-125b-5p, miR-132-3p, miR-143-3p, miR-148a-3p, miR-223-3p, miR-223-5p, miR-29a-3p, miR-34a-5p, miR -424-3p, and miR-424-5p) were overexpressed in post-race marathon runners (199).

The levels of circulating microRNAs can also be modified in response to a treatment. For instance, in a monkey model treated with simvastatin, lower levels of circulating miR-150-5p and miR-451 were observed as well as higher levels of miR-3613-5p compared to the untreated group (200). Another example can be found in mice treated with an antihypertensive agent (captopril) which reduced circulating levels of miR-16, miR-20b, miR-93, miR-106b, miR-223, and miR-423-5p with respect to untreated mice (201).

These investigations reinforce the idea of the potential of circulating microRNAs in the diagnosis of CVD or other types of diseases as well as metabolic states. Moreover, circulating microRNAs are promising biomarkers of treatment response.

5.4. MicroRNAs associated with aging

The expression of many microRNAs could be affected by age. It has been described that the increase in aging-related stress leads to a higher expression of p53. P53, among other functions, has an influence on the Drosha complex, which affects the maturation of microRNAs (202). In addition to this, the microRNA profile (especially circulating microRNAs) also changes with age (203, 204). One of the main studies focusing on the circulating profile of microRNAs associated to aging is the study carried out by Serna *et al*, who studied the circulating profile of microRNAs in 36 subjects (20 centenarians and 16 octogenarians) in peripheral blood mononuclear cells (PMBCs).They discovered that the profile of microRNAs of centenarians was more similar to the profile of young adults than that of the octogenarians (203). These results could suggest that a specific circulating microRNA profile exists that will allow us to predict longevity. However, authors use all PBMCs (peripheral blood mononuclear cells) fraction to identify circulating microRNAs profiles, without differentiate the type of cells. This implies that the different levels of microRNAs observed could be mirroring an age-related change in the cellular composition of the blood and is not reflecting real changes in circulatory microRNAs. Nevertheless, this study is a great example of how microRNA profiling could be used to categorize individuals according to their longevity. In this regard, the profile of circulating microRNAs could also allow us to discriminate between healthy and unhealthy aging, and it could be used to prevent the onset of

the disease before it occurs (205). A recent study in humans and mice showed that circulating levels of miR-34a change with the development of age-related hearing loss (AHL) (206). Another study carried out on Wistar rats showed that the expression of microRNAs changes during aging. The authors specifically found an overexpression in pancreatic islets of miR-34a, miR-124a and miR-383 and lower levels of miR-130b and miR-181a. These changes in the levels of microRNAs could contribute to the failure of pancreatic β-cells resulting in insulin resistance (207). Other studies have shown that circulating miR-34 is positively regulated in age-related macular degeneration (208). Several studies in the cell lines SH-SY5Y and SK-N-SH suggested the possible effects of miR-124 on neuronal apoptosis and autophagy in Parkinson's disease (209). In addition, miR-124 is over-expressed in the skin of elderly people compared to the skin of young people (209, 210). In a study comparing miR-223 levels in young and elderly donors, they observed that these levels decrease in CD4+ T cells in elderly donors with respect to young donors (211). Finally, in human IDH4 fibroblasts, let-7 is related to the expression of p66Shc that is involved in cellular senescence (212). **Table 3** shows a summary of the relationship of the microRNAs and the pathways they modulate with their relationship with aging.

5.5. Modulation mediated by microRNAs of nutrient sensing pathways

The role of microRNAs in aging has not yet been studied in depth as well as the role of microRNAs in the regulation of molecular pathways affected by the aging process. However, some recent studies point to the influence of microRNAs on aging-related processes, such as deregulation of nutrient sensing pathways, immune system dysfunction, cellular damage or age-related diseases (111). **Table 3** shows a summary of the relationship of the microRNAs and the modulating pathways.

5.5.1. Regulation mediated by microRNAs of the IGF1/PI3K/AKT/mTOR pathway

The IGF1/PI3/AKT/FOXO/mTOR pathway is mainly regulated by let-7, a microRNA that targets multiple components of this route, such as the IGF1 receptor or mTOR (167, 168). In *Drosophila melanogaster*, miR-200 and miR-8 are important regulators of PI3K through Zinc finger protein USH/ Zinc Finger Protein, FOG Family Member 2 (*USH/FOG2*) (213). Another study in mouse myoblasts demonstrated that miR-432 is a negative regulator of myoblast proliferation and differentiation directed to the PI3K/AKT/mTOR pathway (214). In a murine model, regulation of this pathway has also been observed by miR-1 (215), a microRNA that is repressed in many cancers and inhibits the growth and proliferation of cancer cells, in addition to promoting apoptosis (215). Other studies show the role of miR-1 in aging. In a mouse model of progeria, it

was found that miR-1 is positively regulated in the liver, independently of the levels of growth hormone (GH) (216). On the other hand, miR-223, a microRNA whose main target is *IGF1R*, participates in the regulation of mast cell apoptosis in rat basophilic leukemic cells (217). Regarding the miR-17-92 cluster, in a mouse model it has been observed that alterations in the levels of miR-17-92 could produce the deregulation of mTOR, producing, in turn, the alteration of the polarity of the Sertoli cells and spermatogenesis (218). The reduction of miR-17-92 cluster expression has also been observed to result in more ROS and DNA damage, which increases age-related damage (219). In liver cancer, the overexpression of miR-145 produces repression of *IRS1* (insulin receptor substrate 1 and 2) and *KIT* (KIT, proto-oncogen receptor tyrosine kinase) (220). The expression of miR-145 also decreases in PBMCs with aging. However, higher expressions of miR-145 and miR-9 are related to lower levels of FOXO (221). In addition, in mouse skeletal muscle cells, treatment with IGF1 results in a reduction of miR-146a levels (222). These studies in animal models highlight the role of microRNAs in the regulation of nutrient sensing pathways, with a potential impact on the aging process. However, human studies analyzing the role of microRNAs in the modulation of these nutrient-sensing pathways and their variations with age are scarce. Olivieri F. *et al* observed a repression of miR-182, miR-223 and miR-142-3p in the skeletal muscle of postmenopausal women. These microRNAs regulate the expression of IGF-1R and FOXO3A, as well as the activation of insulin/IGF-1 signaling through the phosphorylation of AKT and mTOR (223). Another study in humans showed the influence of miR-4458 on the regulation of IGF-1. The levels of miR-4458 are higher and are inversely correlated with the levels of IGF-1R in patients with lumbar disc degeneration (LDD) compared to subjects with damage but without lumbar disc degeneration. These results suggest a relationship between the appearance of LDD with the levels of this microRNA (224). Furthermore, in humans, miR-613, which has c-MET (tyrosine protein kinase MTE) and PI3K/AKT/mTOR as its main targets, is repressed in osteoarcoma and its repression is associated with metastasis in lymph nodes (225). In human melanoma cell lines and melanoma tissues, miR-425 was repressed. The authors suggested that miR-425 could be a tumor suppressor by repressing the PI3K-AKT pathway (226). In aother human cell line, GIST882, microRNA-221 induces apoptosis through KIT/AKT (227). In a bone marrow cell line, it has been shown thatIGF1 can block the processing of miR-34a (228). Finally, in the HOS, KHOS, U2OS and MG-63 cell lines, as well as in osteosarcoma samples from patients, it was observed that miR-16 inhibits cell proliferation via *IGF1R* (229).

5.5.2. Regulation mediated by microRNAs of the AMPK/SIRT1/PGC1α pathway

The AMPK/SIRT1/PGC-1α pathway is also regulated by microRNAs. Some of them can regulate both, the IGF1/PI3/AKT and the AMPK/SIRT1/PGC1α pathways. For instance let-7, which targets different genes of the IGF-1/PI3K/AKT/FOXO/mTOR pathway, also regulates the expression of SIRT1 in human biliary epithelial cells (230). SIRT1 has been shown to be repressed by miR-217 (231), modulating endothelial cell senescence, while miR-33b repressed SIRT-6 and AMPK expression (124). In humans, Kurylowicz A *et al* demonstrated that the repression of SIRT1 is negatively correlated with the expression of miR-22-3p in the adipose tissue of obese individuals and that the overexpression of SIRT7 correlated negatively with miR-125a-5p levels in thin individuals (232). Another study in humans showed the importance of miR-199 as a modulator of SIRT1 and as a biomarker of atrial fibrillation (AF) after coronary bypass surgery (233). Lower miR-199 levels in the brown adipose tissue (BAT) of *Rhesus* monkeys have been associated with age (234). Other microRNAs such as miR-19b, miR-221 and miR-222 are important regulators of PGC1α (235). Interestingly, miR-19b was found to be repressed in octogenarians while centenarians and young people maintained the same level of expression (203). In atherosclerosis, miR-19b, miR-221 and miR-222 can induce endothelial cell dysfunction through the repression of PGC-1α (235). In a transgenic mouse model without miR-455 production in adipose tissue, it has been shown that miR-455 activates AMPK in BAT. These results suggest the importance of this microRNA in the adipogenesis of BAT through the regulation of the AMPK/SIRT1/PGC1α pathway (236). In addition to these results, another study showed that the expression of miR-455 was lower in old mice compared to young mice (237). In the cell lines HEK-293 and HT-29, miR-451 produces an inhibition of AMPK and an activation of mTORC1 (238). In PBMCs, a lower expression of miR-181 (associated to age) modifies the expression of T cells receptor due to DUSP-6 activity, gene regulated by AMPK levels via *ERK* (signal-regulated kinase) (239, 240).

5.6. Effect of CR on the levels of microRNAs

As explained above, there are different strategies that could delay aging or promote healthy aging. These strategies can be based on longevity medications or lifestyle interventions. Regarding to lifestyle interventions, the strategy that has been shown to be effective in different animal models is CR, which results in an increased lifespan and a better state of health (241). The molecular mechanisms by which CR has an effect on aging are not fully understood yet, but microRNAs could play a key role. In rats, CR reduces the expression of miR-144 in cerebrovascular endothelial cells (CMVEC), preventing the reduction of nuclear factor

(erythroid-derived 2)-like 2 (*nrf2*), a regulator of cellular resistance to ROS A low protein diet reduces the expression of miR-124 in the pancreas in pregnant rats (242). However, in old *Rhesus* monkeys, this microRNA was found to be positively regulated by CR in skeletal muscle (243). Other microRNAs that were also differentially modified by CR were miR-451, miR-144, miR-18a, miR-15a, miR-451 (overexpressed) and miR-181a and miR-181b (reduced) (243). In a murine model, it has been observed that miR-425, miR-196, miR-155, miR-150, miR-351, miR-16, let-7, miR-34 and miR-138 are differentially expressed between the control group and the group submitted to a CR (244). Interestingly, as described above, some of these microRNAs that are differentially expressed with CR have been described as regulators of nutrient-sensing pathways (let-7, miR-34, miR-425, miR-16, miR-155, miR-144, miR-451). Other microRNAs have been also described to be modulated by CR. For isntance, CR produces a repression of the miR-17-92 cluster in a mouse model of breast cancer (245). However, the effect of CR on the profile of microRNAs is not yet clear due to lack of studies. For this reason, studies in humans are needed that combine the CR with the modification of the expression of microRNAs. In fact, the potential of microRNAs as biomarkers of aging allows us to suggest the future importance of microRNAs as biomarkers of the effect of moderate CR on human longevity and healthy aging. **Table 3** shows a summary of the relationship of the microRNAs and CR.

5.7. Exogenous microRNAs

In 2012, an study carried out by Zhang L. *et al,* introduced a great controversy about the possible "cross-kingdom" effect of the intake of microRNAs coming from other species and their possible regulatory effect on the gene expression of the persons or animals that ingests them (246). In this study, they detected exogenous plants microRNAs from rice (*Oryza sativa).* They detected specifically high levels of miR-168a in serum of Chinese volunteers whose diet is rich in rice. They also detected it in the serum and tissues of mice after oral intake of rice. Moreover, they observed that this exogenous microRNA was able to repress human *LDLRAP1* (low density lipoprotein receptor adaptor protein 1), a gene related to the regulation of cholesterol in the liver (246). Since the publication of this study, many researches have tried to replicate these results, obtaining different conclusions. Some authors have detected the presence of microRNAs from plants in the serum of patients, such as Yang J. *et al* who detected the presence of miR-2911 from *Lonicera japonica* in the serum of healthy individuals (247). However, this microRNAs was removed of miRBase database due to the fact that it is not a real microRNA, it was found to be a ribosomic RNA. Beatty M. *et al* detected different microRNAs from the order Hypocreales in plasma (248). Chin AR. *et al* detected the presence of miR-159, a microRNA widely conserved

in plants of the order Brassicales in human serum, microRNA that has been observed that can produce an inhibitory effect in the progression of breast cancer (249). Lukasik A. *et al,* performing *in silico* studies, detected microRNAs from plants in milk exosomes of mammals (250). On the other hand, other authors have not been able to detect any type of microRNAs from one species in another (251-253) and technical artifacts have been considered the cause of the detection of exogenous plant microRNAs in human and animal tissues.

The confirmation of whether exogenous microRNAs could be detected in the blood or other biofluids of another species is vital to advance in the understanding of how diet can influence the health status of the individual. The confirmation of these results could change the current conception that we have about the diet since not only would the nutrients have an effect on the organism but the genetic material of the food would also influence their properties. In addition, these discoveries would have a great influence on the development of functional foods and transgenic foods due to the genetic modification that is sometimes made in the brewing of these foods.

MicroRNA	Aging relationship	Regulated pathways	Diet effect	References
let-7	Cellular senescence	IGF1/PI3 /AKT/mTOR , AMPK/Sirtuinas/PGC1α, Inflammation	Differentially expressed in RC	(167, 168, 199, 212, 213, 230, 244)
miR-1	Overexpressed in liver in progeria model	IGF1/PI3 /AKT/mTOR	Inhibited in adipose tissue in mice with high fat diet	(189, 215, 216)
miR-155	Overexpressed in pancreatic β cells	IGF1/PI3 /AKT/mTOR	Differentially expressed in RC	(185, 244)
miR-16	Overexpressed in pancreatic β cells	IGF1/PI3 /AKT/mTOR	Differentially expressed in RC	(201, 229, 244)
miR-34a	Higher circulating levels in macular degeneration and age-related hearing loss. Overexpressed in aging	IGF1/PI3 /AKT/mTOR , AMPK/Sirtuinas/PGC1α, Inflammation	Differentially expressed in RC	(199, 206, 207) (228)
miR-124a	Related to Parkinson disease and overexpressed in old skin	IGF1/PI3 /AKT/mTOR , AMPK/Sirtuinas/PGC1α	Low protein diet reduce its expression in pancreatic cells of pregnant rats	(207, 209, 210, 242)
miR-383	Overexpressed in aging	IGF1/PI3 /AKT/mTOR	Reduced in pancreatic cells after high fat diet	(175, 207)
miR-130b	Inhibited in aging	IGF1/PI3 /AKT/mTOR	Higher circulating levels after polyunsaturated fat intake in women	(183, 207)
miR-181	Inhibited in aging	IGF1/PI3 /AKT/mTOR	Overexpressed in skeletal muscle of monkeys with CR	(175, 176, 207, 239, 243)
miR-223	Reduced in CD4+T in ancient and postmenopausal women	IGF1/PI3 /AKT/mTOR	High protein diet reduce it level	(184, 199, 201, 211, 217, 223)
miR-17-92 cluster	Inhibited in aging	IGF1/PI3 /AKT/mTOR , AMPK/Sirtuinas/PGC1α	Inhibited in RC of breast cancer mouse model	(218, 219, 245)
miR-145	Reduced in PBMCs in aging	IGF1/PI3 /AKT/mTOR	Overexpressed in high fat diet in Wistar rats	(180, 220-222)
miR-19b	Lower levels in octogenarians compared to young and centenarians	AMPK/Sirtuinas/PGC1α	Higher circulating levels after polyunsaturated fat intake in women	(183, 203, 235)
miR-451	Overexpressed in old monkeys	IGF1/PI3 /AKT/mTOR , AMPK/Sirtuinas/PGC1α	Overexpressed in skeletal muscle of monkeys with CR	(200, 238, 243, 244)
miR-142	Inhibited in postmenopausal women	IGF1/PI3 /AKT/mTOR	Overexpressed in high fat diet in mice	(175, 176, 223)
miR-182	Inhibited in postmenopausal women	IGF1/PI3 /AKT/mTOR	Overexpressed in high fat diet in mice	(176, 223)
miR-144	Overexpressed in skeletal muscle in old rhesus monkeys	IGF1/PI3 /AKT/mTOR , AMPK/Sirtuinas/PGC1α	Inhibited in CR in CMEV in rats	(243, 254)
miR-221	Inhibited in aging	AMPK/Sirtuinas/PGC1α	Higher circulating levels after polyunsaturated fat intake in women	(183, 193, 198, 235)

Table 3: Summary of the reported relationships between microRNAs, nutrient sensing pathways, healthy aging and diet

HYPOTHESIS

The hypothesis proposed in this thesis is that the intake of typical Mediterranean foods as well as a CR based on a Mediterranean Diet will promote healthy aging through the modification of microRNAs modulating nutrient sensing pathways such as IGF1/PI3K/AKT/FOXO/mTOR and AMPK/Sirtuins/PGC1-1α. In addition, this project analyzes the existence of exogenous plant microRNAs in human plasma after the consumption of typical Mediterranean foods.

OBJECTIVES

1. To study the effect of the intake of two usual foods of the Mediterranean Diet on the expression of microRNAs associated with the aging process. Study of exogenous microRNAs from olive oil and beer in human plasma.
   a) Study of **exogenous microRNAs** in human plasma. Bioavailability and conservation of microRNAs after the processing of olive oil and beer.
   b) Study of the effect of olive oil intake on circulating microRNAs**. VOHF study.**
   c) Study of the effect of alcohol ingestion with and without alcohol on circulating microRNAs and microRNAs from macrophages associated with aging. **MiRoBEER study.**

2. To study the effect of a CR based on a Traditional Mediterranean Diet, physical activity and behavioral therapy against the same Traditional Mediterranean Diet without CR on variables associated with a healthy aging in the individuals of the population **PREDIMED-PLUS study.**
   a) Study of cognitive impairment and perception of quality of life throughout the intervention and follow-up associated with the intervention by measuring parameters related to healthy aging.
   b) Study of changes in the expression of circulating microRNAs and of and macrophages involved in the regulation of the immune system, pathways of nutrients and aging.

3. Analysis of the molecular mechanisms of action of microRNAs associated with cardiovascular health and healthy aging detected in Objective 1 and 2 through the use of bioinformatics and molecular biology techniques.

MATERIALS AND METHODS

1. Material

L-929 cells (ATCC® Number: CCL-1™) were used to activate blood monocyte-derived macrophages. For cell culture, we used DMEM medium with 4.5 g/L Glucose (Cultek, Madrid, Spain) for L-929 cells and RPMI 1640 with L-Glutamine (Cultek, Madrid, Spain) for human PBMCs. Both of them were supplemented with a mix of 100 U/ ml of penicillin, 100 µg/ml of gentamycin and 100 U/ml of streptomycin (Lonza, Basilea, Switzerland). Moreover, culture cell medium was supplemented with 10% of fetal bovine serum (FBS) (Gibco, Invitrogen, Carlsbad, California). Other materials used in cell culture were phosphate buffered saline (PBS) (Gibco, Invitrogen, Carlsbad, California), trypsin with ethylenediaminetetraacetic acid (EDTA) (Lonza, Basilea, Switzerland) and dimethyl sulfoxide (DMSO) (Scharlau, Barcelona, Spain).
Other general material used was RNA free water (Sigma), ethanol (Scharlau, Barcelona, Spain) and chloroform (Scharlau, Barcelona, Spain).

2. Study populations

2.1. Exogenous microRNAs

Five healthy volunteers aged 25–35 years old were recruited at IMDEA Alimentación. All volunteers consumed 40 ml of EVOO after 12 hours fasting. Blood samples were collected at 1, 2, 4 and 6 hours after EVOO ingestion by venous puncture. Intervention and blood collection were carried out at Hospital Ramón y Cajal (Madrid, Spain). The Ethics Committee of the institute approved the intervention protocol, and all volunteers provided written informed consent. All procedures were in accordance with the 19564 Helsinki Declaration and its late amendments.

2.2 VOHF Study

VOHF study is a postpandrial, randomly, controlled cross-over study carried out between April and September 2011 in Hospital Universitari Sant Joan de Reus. 12 healthy volunteers, 6 men and 6 women (aged 20–70 years old) were recruited through a volunteer centre database. Participants were considered healthy according to a physical examination and routine laboratory tests. Volunteers participated in three one-day experimental sessions and received, after an overnight fast (12 hours), a single ingestion of 30 mL of each phenol-enriched EVOO and the three treatment conditions were separated by a 1-week washout period. Venous blood was

collected at baseline (0 h) and at several time points after EVOOs intake (1, 2, 4 and 6 hours after the ingestion). Three intervention periods were included with three different polyphenols-enriched EVOO compounds containing 250 (L-EVOO), 500 (M-EVOO) and 750 (H-EVOO) mg/Kg of EVOO-derived polyphenols (255). A summary of the intervention and collection times are in **figure 7**. Phenolic composition of each functional EVOO is shown in **table 4.**

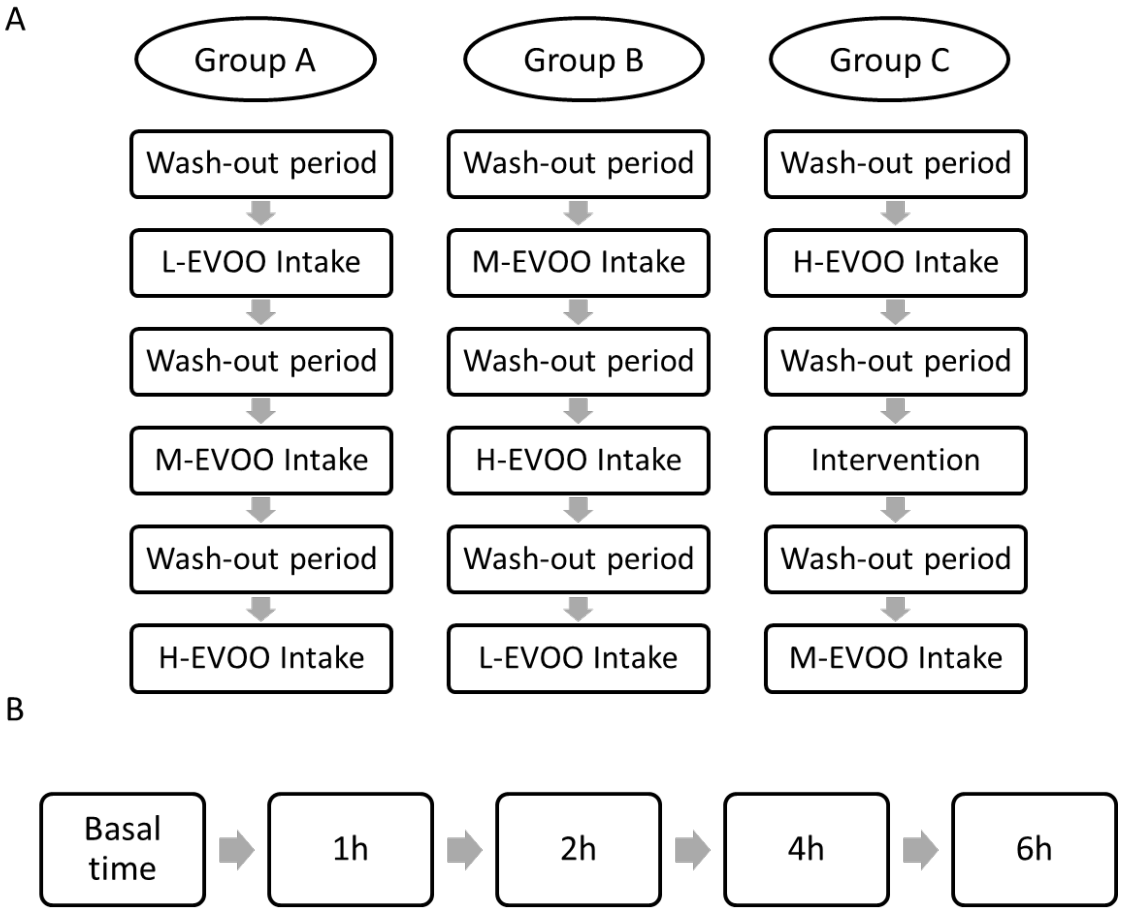

Figure 7: Flow-chart of intervention (a) and samples collection (b) in VOHF study (modified of figure 1 of Valls R-M *et al* (255)).

The intervention was carried out according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Clinical Research Ethical Committee of the Hospital Universitari Sant Joan de Reus (Ref 09-02-26/2proj2), Spain and was registered at ClinicalTrials.gov (Identifier: NCT01347515).

Table 4: Composition of functional EVOO used in VOHF study (modified of supplementary table 1 of Valls R-M *et al* (255)). All data are shown in mg/ 25 ml of EVOO

Compound (mg oil ingested dose)	L-EVOO	M-EVOO	H-EVOO
Hydroxytyrosol	0.21	0.29	0.37
3,4-DHPEA-AC	0.16	0.60	0.90
3,4-DHPEA-EDA*	1.51	4.67	8.42
3,4-DHPEA-EA*	0.36	0.71	1.18
Total Hydroxytyrosol derivatives	**2.23**	**6.27**	**10.87**
p-hydroxybenzoic acid	0.00	0.02	0.03
Vanillic acid	0.02	0.08	0.10
Caffeic acid	n.d	n.d	n.d
Rosmarinic acid	n.d	n.d	n.d
Total phenolic acid	**0.02**	**0.10**	**0.13**
Pinoresinol	0.27	0.19	0.19
Acetoxipinoresinol **	6.95	5.98	5.81
Total lignans	**7.22**	**6.17**	**6.00**
Luteolin	0.17	0.28	0.57
Apigenin	0.06	0.08	0.10
Naringerin	n.d	n.d	n.d
Eriodictyol	n.d	n.d	n.d
Thymusin	n.d	n.d	n.d
Xanthomicrol	n.d	n.d	n.d
7-methylsudachitin	n.d	n.d	n.d
Total flavonoids	**0.22**	**0.35**	**0.67**
Thymol	n.d	n.d	n.d
Carvacrol	n.d	n.d	n.d
Total monoterpenes	n.d	n.d	n.d
Total phenols HPLC-MS/MS	**9.69**	**12.89**	**17.67**

* Quantified with calibration curve of hydrocytyrosol ** Quantified with calibration curve of pinoresinol n.d. non-determined

2.3 MiRoBeer study

The miRoBEER study is a controlled crossed-over nutritional intervention study in which 2 different types of beer were used (alcohol and non-alcoholic beer). Male volunteers consumed both types of beer for 14 days, with a 1-week washout period between interventions. Volunteers were provided with the amount of beer needed at the start of each intervention, divided into individualized daily doses. The daily dose of beer was 500 ml, an amount that has been shown to be within a moderate consumption range and, therefore, safe for the volunteers according to the "Dietary Guidelines for Americans 2015-2020," U.S. Department of Health and Human Services and U.S. Department of Agriculture". Blood and urine samples were collected at the beginning and at the end of each intervention and washout period following the flow diagram in **figure 8**. The Ethics Committee approved the protocol for the recruitment, intervention and

follow-up of the miRoBEER project (Ref: IMD: PI-016.) and all procedures were done according to the Declaration of Helsinki. All volunteers signed an informed consent to participate in the study.

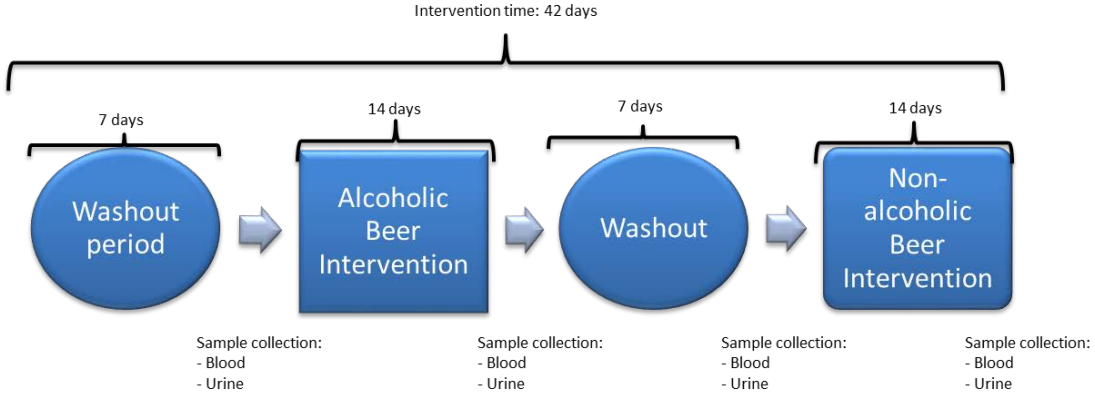

Figure 8: Intervention design flow-chart for miroBEER study

Seven participants were recruited with the following inclusion criteria:

- Male
- Age between 25-65 years
- Non-smoker
- 2 or more cardiovascular risk factors:
    - Family history of Early Coronary Disease: documented myocardial infarction or sudden death before 55 years of age in first-degree male relatives or before 65 years of age in first-degree female relatives.
    - High blood pressure (≥ 130/85 mm Hg) or taking antihypertensive medication.
    - Body mass index (BMI) ≥ 25 kg / m2
    - HDL-cholesterol ≤ 40 mg / dl, LDL-cholesterol ≥ 160 mg / dl without taking medication for hypercholesterolemia.
    - Increased triglycerides (> 150 mg /dL) without taking medication for its reduction.

Exclusion criteria were:

- Documented history of CVD, such as coronary disease (angina, myocardial infarction, coronary angioplasty techniques or abnormal Q on the electrocardiogram (ECG)), stroke (both ischemic and hemorrhagic, including transient vascular accidents), and symptomatic peripheral arteriopathy diagnosed by imaging techniques.
- Serious medical illness that prevents the patient from participating in a nutritional intervention study.

- Immunodeficiency or HIV infection.
- Toxic abuse or chronic alcoholism.
- Impossibility to continue the intervention for religious reasons or for mastication or swallowing disorders.
- History of food allergy with hypersensitivity to any of the components of beer.
- Participation in pharmacological clinical trials or taking any investigational drug in the last year.
- Volunteers admitted to institutions.
- Diabetes.
- Chronic illness, without autonomy, unable to walk or without a fixed postal address.
- Illiteracy.

The participants in the study also had to fill-in the following questionnaires at each control point:

- 3-day diet registry.
- CAGE questionnaire to detect alcohol risk consumption (256).
- Intervention compliance questionnaire.

Participants' recruitment flowchart is present in **figure 9.**

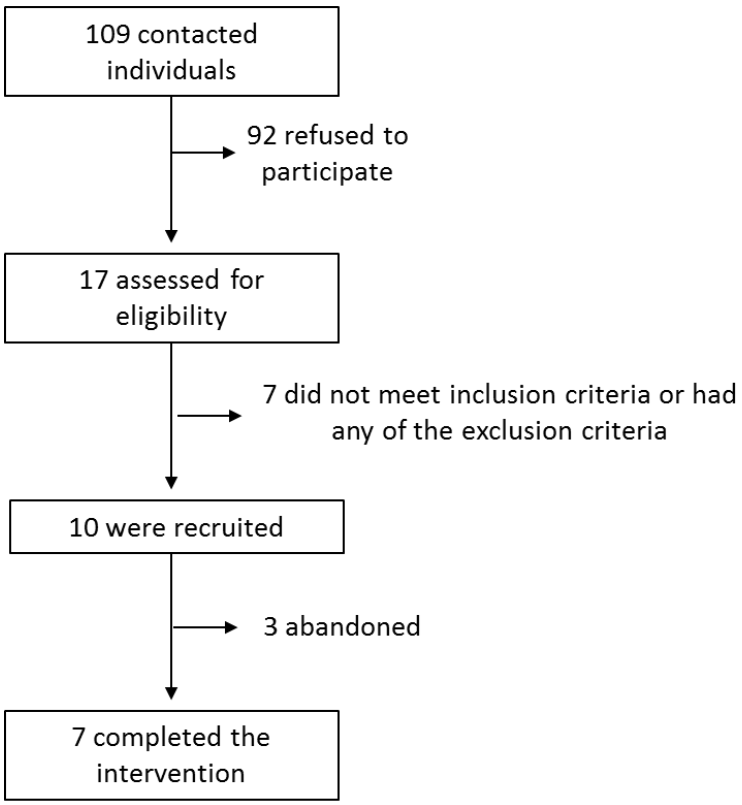

Figure 9: Participants recruitment flow-chart for miroBEER study

The intervention was performed with two washout periods of 7 days each and two intervention periods (alcoholic and non-alcoholic beer) of 14 days each **(figure 8)**. During the washout period, the participants were not allowed to consume any type of fermented beverages while in the intervention period participants only could consume the beer proportionated by the clinical trial. In every collection time, blood and urine samples were collected. Blood was collected by venous puncture. Recruitment, intervention and sample collection was carried out at IMDEA Food.

2.4. PREDIMED-PLUS

PREDIMED-PLUS project is a multidisciplinary, multicentre study formed by a consortium of 23 research groups from more than 7 autonomous communities that has recruited a total of 6874 volunteers. Our group is a recruitment and intervention group that has recruited 174 participants. The protocol of recruitment, intervention and monitoring of the PREDIMED-PLUS project has been approved by the Clinical Research Ethics Committee of the Sant Joan de Reus University Hospital (Ref .: 13-07-25 / 7proj2) and by the Ethics Committee of the IMDEA Food Research (Ref: IMD: PI012). This study will last for 6 years but this thesis will only take the corresponding data up to 1 year of intervention.

Recruitment of volunteers has been carried out from April 2015 to December 2016. Our recruitment node has recruited 174 participants with the collaboration of the Primary Care Centres of the Community of Madrid. The volunteers were recruited with the help of medical and nursing staff belonging to different centres. These health centres have been:

- Buenos Aires Primary Care Centre
- Bustarviejo Primary Care Centre
- Colmenar Viejo Primary Care Centre
- Fuencarral Primary Care Centre
- Ibiza Primary Care Centre
- Aquitania Primary Care Centre
- Hospital La Paz

The recruitment process began with a 4-week run-in period that included 2 individual visits where the commitment of the patients and their ability to complete the study were evaluated. The visits carried out during the screening period were:

• Selection visit 1: During this visit, the potential participant received the information of PREDIMED-PLUS study and the inclusion criteria were checked. The participant was asked to

keep a weight, waist and hip perimeter record of 4 weeks. The participant was instructed to perform the measurements and was given a tape measure. A 3-day dietary record was also requested. On this visit the signature of the informed consent was requested.

• Selection visit 2: a telephone call was made to the participant to follow up on the weight, waist, hip and diet registration tasks and he/she was reminded of the date of the next visit.

• Selection visit 3: An evaluation was made that included: i) the verification of the correct performance of weight, waist, hip and diet recording as a way to evaluate their ability to follow the intervention instructions throughout the study and its implication and ii) the inclusion and exclusion criteria. On this visit, his/her inclusion was decided.

The inclusion criteria were:

- Men between the ages of 55 and 75 and women aged between 60-75 years.
- BMI ≥ 27 and <40 kg / m2.
- Meet at least 3 of the following criteria for metabolic syndrome:
    - Waist circumference ≥102 cm (men) and ≥88 cm (women).
    - Triglycerides> 150 mg / dL or taking medication for its treatment.
    - HDL cholesterol ≤40 in men and ≤50 mg / dL in women or taking medication for its treatment.
    - High blood pressure (≥130 / 85 mm Hg) or taking medication for its treatment.
    - High fasting blood glucose (≥100 mg / dL) or taking medication for its treatment.

Exclusion criteria:

- Illiteracy or inability/unwillingness to give written informed consent or communicate with study staff.
- Institutionalization (the participant is a permanent or long-stay resident in a care home).
- Documented history of previous CVD, including: angina; myocardial infarction; coronary revascularization procedures; stroke (ischemic or hemorrhagic, including transient ischemic attacks); symptomatic peripheral artery disease that required surgery or was diagnosed with vascular imaging techniques; ventricular arrhythmia; uncontrolled AF; congestive heart failure (New York Heart Association Class III or IV); hypertrophic myocardiopathy; and history of aortic aneurism >=5.5 cm in diameter or aortic aneurism surgery.
- Active malignant cancer or history of malignancy within the last 5 years (except nonmelanoma skin cancer).
- Inability to follow the recommended diet (for religious reasons, swallowing disorders, etc.) or to carry out PA.

- A low predicted likelihood to change dietary habits according to the Prochaska and DiClemente Stages of Change Model.
- Inability to follow the scheduled intervention visits (institutionalization, lack of autonomy, inability to walk, lack of stable address, travel plans, etc.).
- Inclusion in another program that provides advice on weight loss (> 5 kg) in the six months before the selection visit.
- History of surgical procedures for weight loss or intention to undergo bariatric surgery in the next 12 months.
- History of small or large bowel resection.
- History of inflammatory bowel disease.
- Obesity of known endocrine origin (except for treated hypothyroidism).
- Food allergy to any component of the Mediterranean diet.
- Immunodeficiency or HIV-positive status.
- Cirrhosis or liver failure.
- Serious psychiatric disorders: schizophrenia, bipolar disorder, eating disorders, or depression with hospitalization within the last 6 months.
- Any severe co-morbidity condition with less than 24 months' life expectancy.
- Alcohol abuse or addiction (or total daily alcohol intake >50 g) or drug abuse within the past 6 months.
- History of major organ transplantation.
- Concurrent therapy with immunosuppressive drugs or cytotoxic agents.
- Current treatment with systemic corticosteroids.
- Current use of weight loss medication.
- Concurrent participation in another randomized clinical trial.
- Patients with an acute infection or inflammation (e.g. pneumonia) will be allowed to participate in the study 3 months after resolution of their condition.
- Any other condition that may interfere with adherence to the study protocol.

The final number of volunteers recruited at the end of this period was 174 patients. Participants' recruitment flowchart is present in **figure 10.**

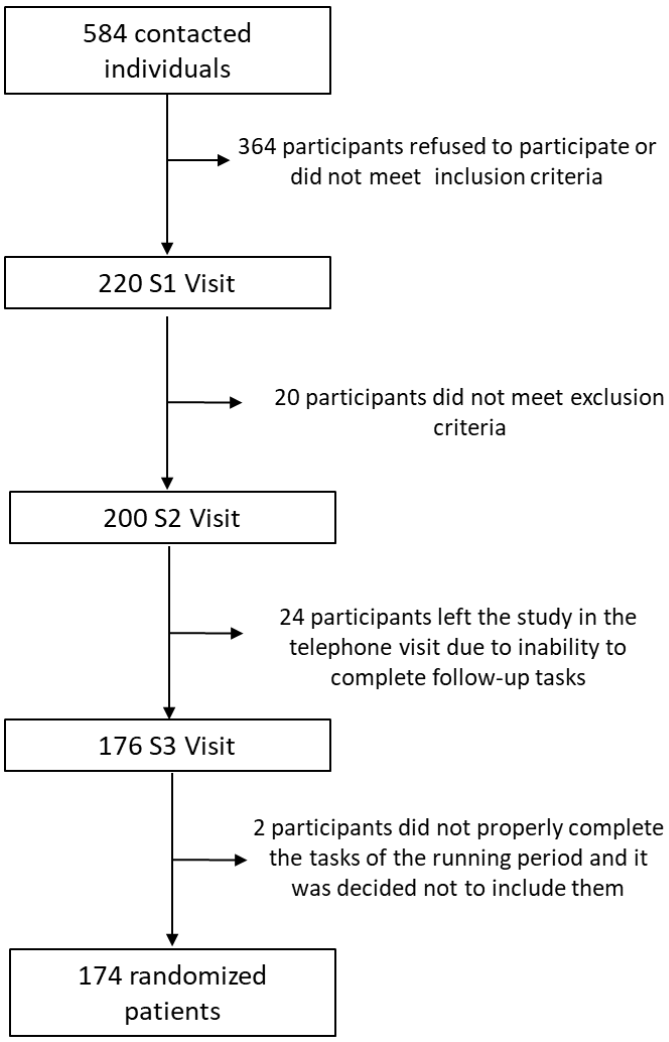

Figure 10: Participants recruitment flow-chart for PREDIMED-PLUS study

The recruited volunteers were randomly divided into a control group or an intervention group. Randomization was carried out with a software application using a multiple randomization method stratified by age and sex. The members of the same family unit were assigned to the same group. The family unit group was randomly selected by randomizing one member of the family unit and assigning the other members of the family to the same group.

The control group received general advice on adherence to MD without any objective of weight loss or intervention with PA. The control protocol included an individual visit and a group session every 6 months in the first year and one individual visit every year a group visit every 6 months from year one up to the end of the study.

In the intervention group, an intensive intervention was carried out on participants the lifestyle with a hypocaloric diet based on T2DM, PA and behavioral therapy, with a goal of 8% weight loss to be reached in the first 6 months of interventions. The intervention protocol included an individual visit, a reinforcement telephone call and a group visit per month during the first year.

From the first year, individual visits were every 3 months with a call for intermediate reinforcement and group visits were held on a monthly basis.

During the study, different questionnaires of food consumption, cognitive assessment, quality of life, PA and anthropometric measurements were collected. These questionnaires are:

- 3-day dietary record
- Food consumption frequency questionnaire (FFQ) (137 items).
- Adherence questionnaire to a MD of 14 and 17 items.
- PA and physical condition questionnaires:
    - Minnesota PA questionnaire in the free time.
    - PAR-Q, RAPA Questionnaires (RAPA1 and RAPA2).
    - NHS sedentarism questionnaire.
    - Chair Stand Test.
- Accelerometry.
- Electrocardiogram.
- Measurement of blood pressure.
- Cognitive-neuropsychological tests:
    - Mini-Mental State Examination (MMSE).
    - Clock-drowing test, verbal and phonological verbal fluency (animals + P) and Digit Span test (WAIS-III)
    - Trail Making Test (TMT)
    - Psychopathological Questionnaire:
    - Beck Depression Questionnaire (BDI-II)
    - Multidimensional scale of control locus on weight and diagnostic criteria of eating disorders (ACT)
    - Health-Related Quality of Life Questionnaire (SF-36)

At the basal time, as well as at 6 and 12 months from the time of patient recruitment, a blood sample collection was carried out, as well as a follow-up of clinical events and progression of health status. Blood collection was performed by venous puncture at primary care centers or at IMDEA Food.

2.5 Data and samples management

The collected samples were stored in a biobank according to the law 14/2007 of bio-sanitary research and by Royal Decree RD 1716/2011 on the operation of biobanks. In addition, a collection of our own samples has been made and stored in our research center in order to use

them for the analyses. The collection of samples and their associated data were registered in a file in the Spanish Data Protection Agency according to the UE regulation 2016/976. Data management and storage are in accordance to RGPD (05/25/2018), Ley Orgánica 15/1999, de 13 de diciembre de protección de datos de carácter personal and LEY 14/2007 de 3 de julio, de Investigación Biomédica.

3. Plasma, urine and PBMCs collection

3.1. Plasma and urine collection

Fasting blood samples were taken from the participants of all studies in each collection time-point. Blood was collected in a 10 ml Vacutainer tubes with K_2EDTA anticoagulant. Blood samples were centrifuged at 1500 x g for 15 minutes and plasma were finally recovered. A spot urine sample was collected in the morning (08:00). All samples were stored at -80°C until processing. Biochemistry analyses in miRoBEER and PREDIMED PLUS were carried out by CQS laboratories (Madrid. Spain).

3.2. PBMCs isolation and macrophage selection

Fasting blood samples were collected in a 10 ml Vacutainer tubes with K2EDTA anticoagulant. Total blood was mixed with 10 ml of complete RPMI complete medium. The sample was passed to another tube with 10 ml of LYMPHOPREP™ (STEMCELL Technologies. Grenoble. France). The mixture was centrifuged at 900g during 40 minutes and the intermediated cell layer containing the PBMCs was carefully collected. The purified PBMCs were washed 3 times with PBS. Two different protocols were carried out depending of the study. In miroBEER study, PBMCs were cultured in RPMI complete medium in a non-treated plate during 4 hours and incubated at 37ºC 5% $C0_2$ conditions. After that, non-adherent and adherents cell (macrophages) were picked up separately and cryopreserved in RPMI medium with 10% DMSO and 10% FBS. Cryopreserved cells were stored in liquid nitrogen until its use. Adherent cells were used without any other processing. For PREDIMED-PLUS Study, all PBMCs were cryopreserved in cryopreservation medium. We defrosted cells and they were cultured in non-treated plates with RPMI complete medium during 24 hours. After that, we separated adherent and non-adherent cells. Non-adherent cells were processed, and adherent cells were cultured during 6 days with activating medium (complete medium with 15 % of L-929 culture medium filtered) to promote the

conversion of monocytes into macrophages and their activation. Only cells adhered to the plate were picked and processed.

4. MicroRNAs extraction

4.1 Oil

Two methods were carried out. In one method, 100 µl of EVOO was extracted using miRCury RNA Extraction Kit – Cell & Plants (Exiqon A/S, Vedbaek, Denmark) with minor modifications: Addition of Additive Buffer, and MS2-RNA-carrier (Roche, Barcelona, Spain) to optimize sample homogenization and microRNA precipitation.

In the second method, 30 ml of EVOO were centrifuged at 16,000g during 10 minutes at room temperature following the procedure reported by Rossi and colleagues for DNA purification adapted to microRNA purification (257). After that, we discarded the supernatant and kept the pellet. Then, we added 500 µL of TRIPURE (Roche Life Science, Barcelona, Spain) and continued the extraction according to the manufacturer's instructions with minor modifications. To optimize the precipitation of microRNAs we incubated samples with the TRIPURE during 24 hours at -80ºC before phase separation, we also increased the isopropanol precipitation period to 24 hours at -80ºC and we washed samples with 85% ethanol instead of 70% ethanol. For sequencing purposes, three samples were extracted simultaneously, pooled and concentrated to reach a concentration of ≈ 30 ng/µl using a CentriVap Centrifugal Vacuum Concentrator (Labconco).

4.2. Beer

500 mL of beer was subjected to lyophilisation in a freeze dryer LYOBETA-15 (Telstar). 200 mg of lyophilized beer was diluted in 400 µL of RNase free water and used for RNA isolation. Total RNA was isolated using TRIPURE (Roche Life Science, Barcelona, Spain) according to the manufacture´s instruction with the same modifications described above.

4.3. Milk

5 ml of fresh, non-pasteurised milk was used for RNA isolation using TRIPURE with the same protocol and modifications used for beer and olive oil. All samples were diluted in 50µl of RNAse free water.

4.4. Plasma

Plasma microRNAs were isolated with miRCURY™ RNA Isolation Kit – Biofluids (Exiqon A/S, Vedbaek, Denmark) from 200 µL of plasma. In VOHF and miroBEER study 5fmol of two single strand spike-in controls were added to check the quality of the extraction. These spike-in controls were ce-miR-34 and ce-miR-238. However, during the extraction process these microRNAs were degraded due to their single-strand conformation. In PREDIMED-PLUS study the spike-in controls used were 75fmol ce-miR-67 and ce-miR-34. In this case, we used double strand spike-in controls to avoid degradation. All samples were diluted in 50µl of RNAse free water.

4.5 Cellular fraction

For isolation of microRNAs from the cellular fraction, we used miRCURY ™ RNA Isolation Kit -Cell & Plant (Exiqon A/S, Vedbaek, Denmark, No. 300121). As well as for the 5 plasma samples, we used the same spike-in controls described before for miroBEER samples and PREDIMED-PLUS samples. All samples were diluted to 50 µl. RNA was quantified and integrity was assessed with a Nanodrop 2000 spectrophotometer (Thermo Scientific, Grand Island, NY).

5. Next generation sequencing (NGS)

LifeSequencing (Valencia, Spain) performed NGS in plasma samples using the Ion PGM System (LifeTechnologies, California, United States). Sequencing adapters were trimmed and only reads with a mean Phred quality score higher than 20 were retained. The Phred quality score is a logarithmic measure of the base-calling error probability. A Phred value ≥20 indicates a ≥ 99% accuracy in the base-calling. Reads were aligned end-to-end against the reference microRNA sequences running the Bowtie2 aligner with the very sensitive option and setting the length of the seed substrings parameter –L to 6 for microRNA's detection. Reads aligning against the MS2-RNA carrier were removed and runs from duplicated reads were combined. The remaining reads were aligned against a reference set build upon the Viridyplantae clade microRNAs, to which *Olea europaea* belongs. We allowed a maximum of 3 mismatches in the alignment procedure. Sequencing data have been deposited in the GEO database (GSE76735).

6. MicroRNAs selection

For VOHF and miroBEER study we selected a panel of 53 microRNAs related to CVD using prediction algorithms such as miRWalk (258) and miRbase (259) and a literature search **(table 6).** For PREDIMED-PLUS study we selected a list of genes which are related to IGF-1/AKT/FOXO/mTOR pathways and AMPK/SIRT1/PGC1α pathway. Then, we used different microRNAs prediction algorithms to select microRNAs that target most of the common genes in these pathways. The used algorithms were Target Scan (using a threshold of PCT > 0.8 and a context+ score < 0.4 and a context score percentile rank > 80%), miRWalk (using the list of genes validated and predicted for at least 7/9 algorithms), miRanda-SUR (using the threshold of SUR < 0.5, energy = -14 and an alignment score threshold of 120). In addition to this, we used bibliography resources to select a list of potential microRNAs and, after an enrichment analysis, we finally selected a panel of 53 microRNAs with 3 spike-in controls **(figure 11).** The selected microRNAs are shown in **table 5**

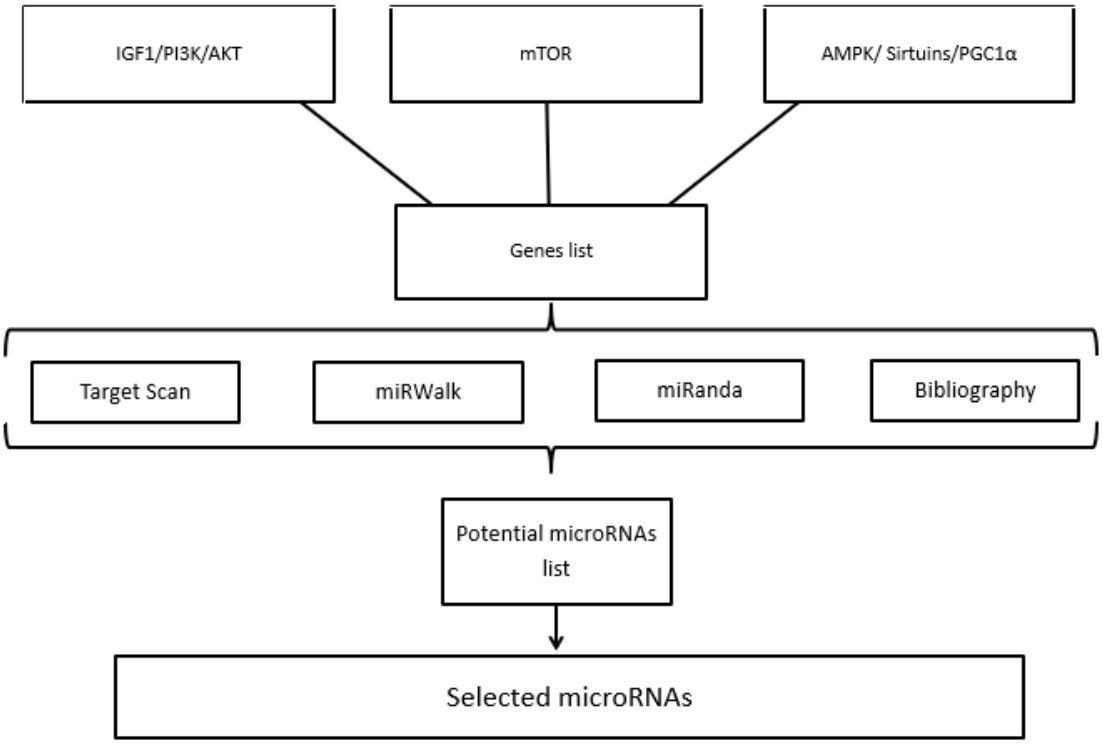

Figure 11: Flow-chart microRNAs selection for PREDIMED PLUS study

Table 5: MicroRNAs selected for PREDIMED-Plus study

cel-miR-67	hsa-miR-21	hsa-miR-15b	hsa-miR-221
cel-miR-34	hsa-miR-22	hsa-miR-182	hsa-miR-27b
cel-miR-54-5p	hsa-miR-98	hsa-miR-183	hsa-miR-29b
dme-miR-7	hsa-miR-138	hsa-miR-195	hsa-miR-301
hsa-let-7a	hsa-miR-145	hsa-miR-19a	hsa-miR-30b
hsa-miR-16	hsa-miR-152	hsa-miR-20a	hsa-miR-30c
hsa-miR-30d	hsa-miR-424	hsa-miR-106a	hsa-miR-181b
hsa-miR-30e	hsa-miR-449	hsa-miR-130a	hsa-miR-196b
hsa-miR-33a	hsa-miR-454	hsa-miR-133a	hsa-miR-200a
hsa-miR-34a	hsa-miR-497	hsa-miR-135a	hsa-miR-200c
hsa-miR-34c	hsa-miR-506	hsa-miR-148b	hsa-miR-216a
hsa-miR-373	hsa-miR-92a	hsa-miR-181a	hsa-miR-302c
hsa-miR-520a	hsa-miR-30a-5p	mmu-miR-93	mmu-miR-153
hsa-miR-17-5p	hsa-miR-520c-3p	mmu-miR-96	mmu-miR-124a

miRNA	Function	Olive oil component	up/downregulated	References
cel-miR-238	spike in			
cel-miR-34	spike in			
cel-miR-54	spike in			
let-7e	Muscle glucose metabolism/Hypertension (let-7e)	Oleic acid (let-7f)	up	J Nutr. 2014 May;144(5):575-85
miR-103	Adipocyte/hepatic glucose metabolism	Conjugated linoleic acid		PLoS ONE 5, e13005.
miR-106b	Reverse cholesterol transport	Palmitic acid	up	J Nutr. 2014 May;144(5):575-85
miR-107	Adipocyte/hepatic glucose metabolism	Conjugated linoleic acid		PLoS ONE 5, e13005.
miR-10a	Angiogenesis and inflammation	Retinoic Acid	up	*BMC Cancer. 2015 May 2; 15():345.*
miR-10b	Reverse cholesterol transport	Unknown		
miR-122	Lipid metabolism/Heart failure	Oleic acid/vitamin E	up	Mol Med Rep. 2014 Jul;10(1):292-300.
miR-124a	Pancreas development	Unknown		
miR-125	Angiogenesis and inflammation	Vitamin E (miR-125a)	up	FEBS Lett. 2008 Oct 15;582(23-24):3542-6.
miR-126	Angiogenesis and inflammation/Heart failure/Coronary arery disease	HFD	up	Mol Med Rep. 2017 Sep;16(3):3061-3068.
miR-129-5p	Heart failure	Unknown		
miR-130	Adipocyte glucose metabolism	PUFA	up	*J Nutr Biochem. 2015 Oct; 26(10):1095-101.*
miR-133	Cardiac hyperthrophy and fibrosis/coronary artery disease	Unknown		
miR-143	Adipocyte/Hepatic glucose metabolism	Conjugated linoleic acid		PLoS ONE 5, e13005.
miR-145	Endothelial function/hepatic glucose metabolism	*Olea europea* extract	up	J Cancer Res Clin Oncol. 2012 Nov;138(11):1831-44
miR-146a	Angiogenesis, inflammation and pancreas development	hydroxytyrosol	down	Int Immunopharmacol. 2017 Feb; 43():147-155.
miR-155	Angiogenesis and inflammation/Coronary artery disease	PUFA/Vitamin D	down	Int J Mol Sci. 2017 Jan 29; 18(2):./ J Immunol. 2014 Mar 1; 192(5):2349-56.
miR-15a	Insulin production and secretion/caardiomyocyte survival	Unknown		
miR-17	Coronray artery disease	Palmitic acid	up	J Nutr. 2014 May;144(5):575-85
miR-182	Insulin production	Unknown		
miR-184	Insulin production	oleic acid	down	Gene. 2018 Jun 30;661:126-132.
miR18b	Heart failure	PUFA	up	J Nutr Biochem. 2015 Oct; 26(10):1095-101.
miR-192	lipid metabolism	Oleic acid	down	J Nutr. 2014 May;144(5):575-85
miR-199b	Cardiac hyperthrophy and fibrosis	Unknown		
miR-208	Cardiac hyperthrophy and fibrosis/Coronary artery disease	RvD1 (EPA derivate)	up	FASEB J. 2011 Feb; 25(2):544-60.
miR-20a	Coronray artery disease	Unknown		
miR-21	Endothelial function, angiogenesis and inflammation. Cardiac hyperthrophy and fibrosis	olec acid	down	Mol Med Rep. 2014 Jul;10(1):292-300. Hepatology. 2009 Apr;49(4):1176-84
miR-214	CAD	Vitamin D	down	Exp Cell Res. 2016 Nov 15;349(1):15-22
miR-22	Aging/ischaemia	Vitamin D	down	Cell Physiol Biochem. 2017;42(1):145-155.
miR-221/222	Endothelial function, angiogenesis and inflammation	Conjugated linoleic acid	down	PLoS ONE 5, e13005.
miR-223	Muscle glucose metabolism	Unknown		
miR-23a	Angiogenesis and inflammation	Unknown		
miR-24	Insulin production/Angiogenesis and inflammation/muscle glucose metabolism	Vitamin E	down	Oxid Med Cell Longev. 2014;2014:725929.
miR-26b	Reverse cholesterol transport/insulin production	Unknown		
miR-27b	Adipocyte glucose metabolism/angiogenesiis and inflammation	Palmitate	up	J Mol Cell Cardiol. 2016 Jan; 90():38-46.
miR-296-5p	Hypertension	Unknown		
miR-29b	Cardiac hyperthrophy and fibrosis/Insulin secretion/Muscle, adipocyte and hepatic glucos	zinc	down	Proc Natl Acad Sci U S A. 2011 Dec 27; 108(52):20970-5.
miR-30c	lipid metabolism	Oleic acid	up	J Nutr. 2014 May;144(5):575-85
miR-320	cardiomyocyte cell survival/Adipocyte glucose metabolism	Unknown		
miR-328	Cardiac rhythm	PUFA	down	J Nutr Biochem. 2015 Oct; 26(10):1095-101.
miR-33	Lipid metabolism/hepatic glucose metabolism	Unknown		
miR-34a	Pancreas development/hepatic glucose metabolism	Palmitic acid/vitamin E	up	Chin Med J (Engl). 2012 Dec;125(23):4202-8. /Int J Cancer. 2012 Dec 1;131(11):2668-77
miR-375	Insulin production and secretion	Unknown		
miR-423-5p	Heart failure	Unknown		
miR-499	Heart failure/Acute myocardial infarction	Linoleic acid	up	Redox Biol. 2013 Nov 7;2:1-7.
miR-503	Angiogenesis and inflammation	Unknown		
miR-622	Heart failure	Unknown		
miR-7	aging (in vitro)	Linoleic Acid	down	J Dermatol Sci. 2013 May;70(2):88-93.
miR-758	Reverse cholesterol transport	Unknown		
miR-9	Insulin secretion	Unknown		
miR-92a	Angiogenesis and inflammation/Coronary artery disease	Unknown		
miR-96	Insulin secretion	Saturated fatty acids	up	PLoS ONE. 2016;11:e0169039
miR-98	Cardiac hyperthrophy and fibrosis	Unknown		

Table 6 MicroRNAs selected for VOHF and miRoBEER studies

7. Selection of patients of PREDIMED-PLUS study for microRNA analysis

After a year of patient follow-up, a total of 20 patients from the intervention group and 20 patients from the control group were selected who were representative of their group to analyze changes in circulating microRNAs as well as microRNAs in macrophages. For this, the following criteria were:

- Patients who have not abandoned the intervention corresponding to their group.
- Patients in whom blood samples have been collected.
- Weight differences between baseline visit and 1 year
    - Weight loss between baseline and 1-year time in the control group: 0 ± 1kg
    - Weight loss between baseline and 1-year time in the intervention group: - 3.5kg ± 1kg (representative of the mean weight loss in the intervention group throughout the study)

8. Analyses of microRNAs concentration and integrity using bioanalyzer

MicroRNAs extracted from beer (200 mg), milk (5 mL) and EVOO (30 mL) were measured and the integrity analyzed with a Bioanalyzer 2100 (Agilent Technologies, Inc, California, EEUU) using an Agilent Small RNA kit to specifically measure small RNA molecules (4-150 nt). This technique provides an electrophoretic-based automated sizing and quantitation method of small RNAs. Purified samples are separated on a Chip for Electrophoretic Assay and results are shown as electropherograms showing fluorescence intensity vs migration time in the electrophoretic gel. Small RNA concentration is calculated from the area under the curve of the electropherogram and converted to concentration values by normalization with a ladder. The concentration of microRNAs was calculated from the area under the curve corresponding to RNA molecules of 8-40 nt. The ratio of microRNAs/small RNAs was also calculated.

9. Changes in microRNAs expression analysis

MicroRNA levels were analysed by Real-Time quantitative PCR (RT-qPCR). Isolated microRNAs were retro-transcribed with the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystem, Waltham, MA, USA). Before retrotranscription, 5fmol of ce-miR 54 was added to control for retrotranscription variability in VOHF and miroBEER study and 75 fmol in PREDIMED-PLUS study. In plasma samples, 5 µl of RNA were used as template and 50 ng of RNA was used from macrophages samples. Resulting cDNA was pre-amplified with the TaqMan® PreAmp Master Mix (Applied Biosystem, Waltham, MA, USA). Preamplification product was diluted 1:20 and 3 µl of diluted preamplification product was amplified by RT-qPCR with TaqMan OpenArray PCR Master Mix (Applied Biosystem, Waltham, MA, USA) in a TaqMan custom OpenArray chip **(figure 12)** loaded using an OpenArray® AccuFill™ System (Thermo Fisher Scientific; formerly Life Technologies, Grand Island, NY). The RT-qPCR was run in a QuantStudio™ 12K Flex Real-Time PCR System with OpenArray® Block (Thermo Fisher Scientific; formerly Life Technologies, Grand Island, NY). The fold-change in circulating microRNAs levels was measured as relative quantification using the $2^{-\Delta\Delta Ct}$ method comparing each time-point to basal point and was Log_2 transformed. In all cases, a blank sample was added as a negative control.

Figure 12: OpenArray plate used in analysis of microRNA expression. Source: Applied Biosystems

Due to the degradation of spike-in controls added to the samples of VOHF and miroBEER study, the NormFinder algorithm (260) was used to select the best two endogenous reference microRNAs in each case (miR-192 and miR-221 for L-EVOO, miR-221 and miR-320 for M-EVOO and miR-223 and miR-92a for H-EVOO). The quadratic average of the two endogenous controls plus the exogenous ce-miR-54 control was used for normalization. In the case of PREDIMED-PLUS study, the quadratic average of all three spike-in controls was calculated for normalization. Efficiency of all probes was assessed with a calibration curve using a control RNA from plasma. All microRNAs with an efficiency below 80% were discarded. Two non-template controls (NTC) were included in each plate and all microRNAs with CTs over the NTC were also discarded. RT-qPCR was made in duplicate and all microRNAs with >10% of inconsistent technical replicates were discarded.

In the study of exogenous microRNAs, we added 200 ng of the hsa-miR-23a mimic and 200 ng of the single-stranded sequence of the osa-miR168a (*Oryza sativa*) during the extraction process to check the potential loss of endogenous miRNAs of the food during the extraction process.

Subsequently, the samples were processed for the isolation of microRNAs as described above, retro-transcribed with the miScript RT II kit (QIAGEN GmbH, Hilden, Germany). hsa-miR-23a and osa-miR168a were amplified by real-time quantitative PCR (RT-qPCR) with the miScipt SYBER Green master Mix kit (QIAGEN GmbH, Hilden, Germany) in a QuantStudio 12KFlex device (Applied Biosystems, California, USA). Absolute quantification was carried out comparing CT of the samples with and without the exogenous controls. In all cases, a blank sample was added as a negative control.

10. Gene expression analysis

For mRNA quantification, total RNA was reverse transcribed with PrimeScript Reverse Transcription kit (Takara, California, USA) and the resulting cDNA was amplified by RT-qPCR with FastStart Universal SYBR Green Master (Roche, Barcelona, Spain). Specific forward and reverse primers were designed using NCBI gene webpage (https://www.ncbi.nlm.nih.gov/gene), Jellyfish software (261)and PrimerBank. We choose a sequence located in an exon with an amplicon size between 100 and 200 nucleotides. Specific primers were provided by Isogen Lifesciences (Barcelona, Spain) (**table 7**) and efficiency was assessed in all cases with a calibration curve. mRNA levels were calculated using the relative quantification method normalizing with RN18S or RLP0.

Table 7: Primer sequences

Primer name	Sequence
hCD68 Fw	GCTACATGGCGGTGGAGTACAA
hCD68 Rev	ATGATGAGAGGCAGCAAGATGG
hCD14 Fw	CCGCTGTGTAGGAAAGAAGCTA
hCD14 Rev	CGCTTTAGAAACGGCTCTAGGT
hCD16 Fw	CCTCCTGTCTAGTCGGTTTGG
hCD16 Rev	TCGAGCACCCTGTACCATTGA
hCD28 Fw	GTTTGAGTGCCTTGATCATGTGC
hCD28 Rev	GGCGACTGCTTCACCAAAATC
hCD57 Fw	CCTGGCGTGGTCTACTTCG
hCD57 Rev	GCAGGTTGACGGCAAATCC
hRPLP0 Fw	CCTCATATCCGGGGGAATGTG
hRPLP0 Rev	GCAGCAGCTGGCACCTTATTG
18S Fw	TAAGTCCCTGCCCTTTGTACACA
18S Rev	GATCCGAGGGCCTCACTAAAC

11. Beer polyphenols analysis

First, we did a calibration curve of the specific polyphenols of beer: IX and 8-PG. Then, we measured these polyphenols in the original beer samples (alcoholic and non-alcoholic beer) that the patients drank during the study.

The analysis of plasma and urine samples was carried out using 500 µL of plasma or urine. Samples were centrifuged at 15,000 x g for 15 min at 4°C and 353 µL of the supernatant was diluted (1:1) with phosphoric acid 4% and spiked with standard mix (50 nM) as an internal standard. 600 µL were loaded on a 96 well µ-SPE HLB plate, washed with 200 µL of water and 200 µL of 0.2% acetic acid and finally eluted with 60 µL of methanol. Extracted and concentrated plasma samples were purified with a solid-phase extraction using Oasis® HLB µElution plate. This process allows 5-times concentration of the initial sample to improve the polyphenols detection. Analyses were carried out using a Thermo Scientific™ Exactive™ Plus Orbitrap (Thermo Scientific). The results were analyzed using Xcalibur software.

12. *In silico* functional analyses

MicroRNAs significantly modified in, at least, one time-point in each treatment were selected for further functional analyses. Experimental validated targets of these microRNAs were identified using miRWalk tool (258). Predicted targets were analysed with miRWalk comparing 9 algorithms (miRanda, TargetScan, miRDB, PITA, MicroT4, miRMap, miRNAMap and miRWalk) using stringent criteria in all of them. Predicted targets by, at least, 7 algorithms were selected for further analysis. Both, experimental and predicted targets were analysed for functional enrichment analysis of GO annotation terms, KEEG pathways and protein-protein interaction using Babelomics 5 and String Software (262, 263). In addition to this, hierarchical cluster analysis with heat map were carried out using TMeV software (264)

Venn diagrams were done with Bioinformatics and Evolutionary Genomics application using default settings (http://bioinformatics.psb.ugent.be/webtools/Venn/)

13. Statistical analysis

The quantitative variables are expressed as mean ± standard deviation (SD) or standard error of the mean (SEM), while qualitative variables are expressed as frequencies or percentages of the total.

In VOHF study, the changes in microRNAs levels along the postpandrial curve were analysed by repeated-measurements ANOVA with Bonferroni correction for multiple testing. The interaction

with the type of oil was included in the analysis. Two-tailed p value of 0.05 was considered significant.

Missing values were imputed using a multivariate linear regression model. Imputed values accounted for less than 10% of all values.

In miroBEER study, the changes in microRNAs levels between every time point (wash out vs intervention points) were analyzed by t-student test. Missing values were imputed using a multivariate linear regression model. Imputed values accounted for less than 10% of all values. The interaction with the type of beer (alcoholic and non-alcoholic beer) was included in the analysis. Two-tailed p value of 0.05 was considered as significant. In addition to this, we did correlation analyses between microRNAs levels in plasma and macrophages and nutrients intake and health parameters. We used Pearson correlation in parametric variables and Spearman correlation with non-parametric variables. A Saphiro-Wilk normal test was carried out to test the normal distribution of the variables. Two-tailed p value of 0.05 was considered as significant.

In PREDIMED-PLUS study, we used the same methodology than in miroBEER study adding new correlations and associations with biochemical (cholesterol, LDL, HDL, glucose), anthropometric (blood pressure, weight, waist, hip, muscular mass, fat mass, visceral fat), diet (14 points MD questionnaire) and lifestyle parameters (PA) obtained during the different time-points. In addition to this, the comparison between mRNA levels of selected genes at the different time-points and among groups was done with t-student test, considering significant a two-tailed p value of 0.05. We performed a paired significance analysis for microarray (SAM) test with hierarchical clustering of significant microRNAs applying a FDR using TMEV software.

All comparisons were carried out using SPSS program version 24.0.

Statistical significance for enriched GO biological processes and KEGG pathways were analysed with Babelomics 5 and Software String applications applying a False Discovery Rate adjustment for multiple testing correction. Overrepresented KEGG pathways are shown in bar charts representing the $-\log_{10}$ of the p value in the X axis.

RESULTS

1. Exogenous microRNAs

1.1. MicroRNA analysis in EVOO, Beer and milk samples

We first analyzed the presence of microRNAs in the EVOO samples extracted with the miRCury RNA Extraction Kit specific for cells and plants. The results obtained from the bioanalyzer showed a concentration of approximately 40 % of short sequences of 20–40 nucleotides **(figure 12a)**. However, the absolute concentration of short RNAs was very low (2.5 ng/µL). After that, the sample was sequenced and we obtained 1.886.007 reads of 8–71 nucleotides. 78.55 % of these reads were less than 20 nucleotides long, but we found a high number of duplicated reads (duplication rate was higher than 80 %). Duplicated reads were similar sequences with different length that had been detected as different sequences. As a quality control step, we filtered reads with a mean Phred quality score lower than 20 (QC20) and therefore 48.27 % of the reads were removed **(figure 12b and c)**. Reads aligning against the MS2 RNA carrier were also removed. The remaining reads were aligned against microRNAs of the *Viridiplantae* clade identifying 9 different sequences putatively corresponding to *Viridiplantae* microRNAs. However, the number of reads of these sequences was very low. The most concentrated sequence, GTCGTTGTAGTATAGTGG, identical to the gma-miR-6300 from soybean, presented only 16 reads **(table 7)**, although with a good mapping quality score.

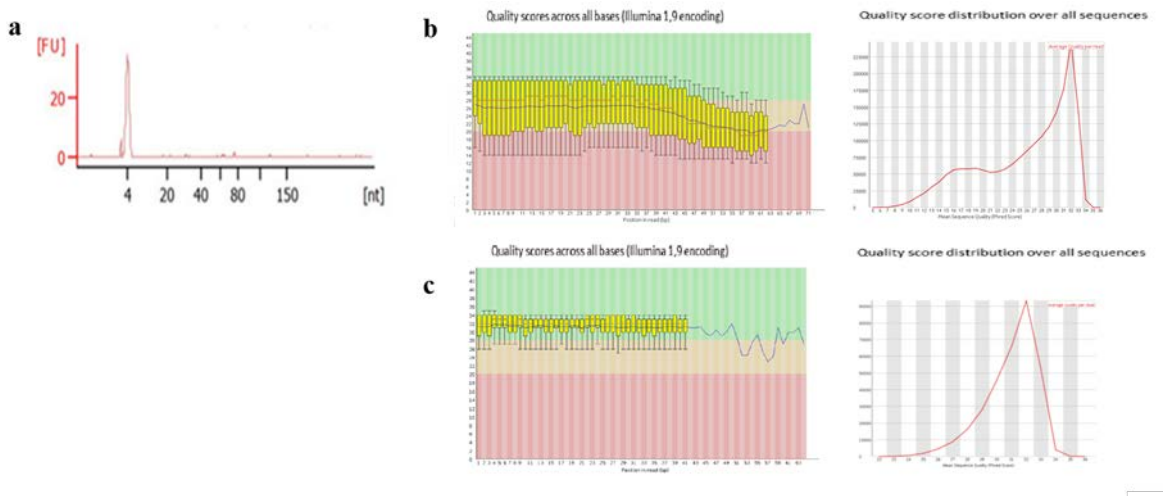

Figure 12: MicroRNAs detection in EVOO a) Electropherogram of 100 µl of EVOO. miRCury Extraction Kit specific for plants was used to extract microRNAs from100 µL of EVOO and the resulting microRNA enriched RNA was analyzed using a bioanalyzer. b) NGS average QC value of the EVOO sequences before quality filtering c) NGS average QC value of the EVOO sequences after filtering by a threshold of 20. [nt], nucleotides; [FU], fluorescence.

Table 7: *Viridiplantae* microRNA sequences identified in EVOO samples by NGS

Sequence ID	miRNA	Read sequence	Mismatches	Reads	Specie
1Q0C3:00392:02459	gma-miR6300	GTCGTTGTAGTATAGTGG	0	16	soybean
1Q0C3:01101:00721	gma-miR396i-5p	TTCCACAGCTTTCTTGAACTG	0	2	soybean
1Q0C3:02727:02326	csi-miR396a	TTCCACAGCTTTCTTGAACTG	0	2	orange tree
1Q0C3:02971:01796	csi-miR396b	TTCCACAGCTTTCTTGAACTG	0	2	orange tree
1Q0C3:02612:02476	gma-miR166h-3p	TCTCGGACCAGGCTTCATTCC	0	1	soybean
3MFN1:02624:01184	gma-miR1529	TTCAAGGAAACACTTAATCTT	0	7	soybean
1Q0C3:02633:00397	gma-miR5675	TAGAGACGACAACCATGCA	2	1	soybean

Then, we repeated the extraction increasing the starting EVOO sample by 300-fold (30 mL) and we included a lyophilized beer sample of 200 mg and a positive control (5 mL of bovine milk). The presence of microRNAs in bovine milk is well documented, although their absorption through the human gut is also under debate (265-268). The extraction was replicated 15 times for EVOO, 12 times for beer and 8 times for milk. The average amount of RNA obtained was 8335 ng ± 3864 ng and 8585 ng ± 3434 ng in 30 mL of EVOO and 200 mg of lyophilized beer, respectively.

The average RNA amount obtained in 5 mL of milk was 4560 ± 905.5 ng **(table 8)**. 260/280 ratios ranged from 1.49 to 1.65, and 260/230 rations were below 0.5, which is indicative of a certain grade of contamination with proteins or solvents.

Table 8: RNA quantification with Nanodrop 2000

Sample	Replicates number	RNA quantification (ng)	260/280	260/230	Starting material
EVOO	15	8335 ± 3864	1.65 ± 0.08	0.25± 0.09	30 ml
Beer	12	8585 ± 3434	1.49 ± 0.04	0.29 ± 0.05	200 mg (lyophilized beer)
Milk	8	4560 ± 905.5	1.51 ± 0.14	0.34 ± 0.19	5 ml

Since Nanodrop measurements do not allow to specifically quantifying microRNAs and we cannot rule out the possibility of an artefactual measurement, we performed bioanalyzer analyses. Bioanalyzer results showed a relative high concentration of small RNAs and microRNAs in the milk sample (48 ng/5 ml) and a microRNA/small RNA ratio of 90 % **(figure 13)**. However, the presence of small RNAs in beer and EVOO samples was lower than expected (20.5 ng/ 200 mg of lyophilized beer and 1.6 ng/30 ml of EVOO, respectively). The ratio of microRNA/Small RNA was 30 % **(figure 13)** and 16 % in beer and EVOO, respectively **(figure 13)**.

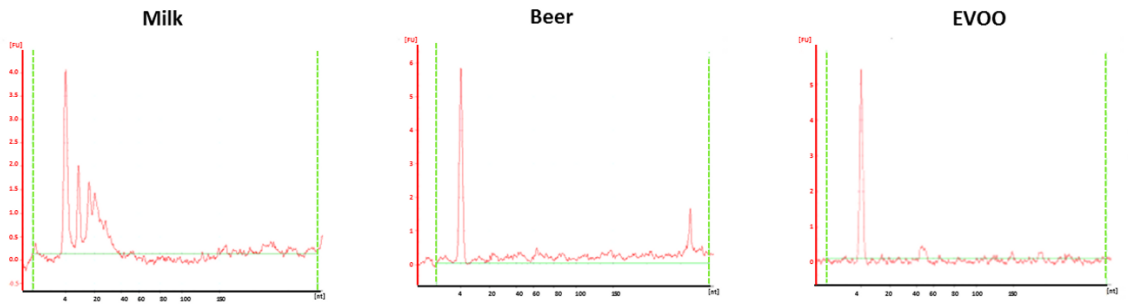

Figure 13: Electropherogram of samples extracted using the phenol-chloroform method. 5 mL of milk, 200 mg of lyophilized beer and 30 mL of EVOO were used to extract microRNA-enriched RNA that was subsequently analyzed using a bioanalyzer. [nt], nucleotides; [FU], fluorescence

To check if microRNAs were lost during the purification procedure, we repeated the EVOO and beer extraction adding two spike-in controls (human miR-23a mimic and plant osa-MIR168a) and analyzed them by RT-qPCR **(figure 14)**. We observed that Cts of samples without the spike-in controls were similar to that of the NTC. However, both sequences were amplified in those samples with the spike-in controls with lower Ct values ranging from 5 to 13 cycles lower than the samples without the spike-ins. These results suggest that our method is useful to purify microRNAs from these samples, but they also suggest that the absence of microRNAs in our samples could not be attributed to the extraction method. However, we should keep in mind that our methods could be further optimized to yield higher amounts of microRNAs.

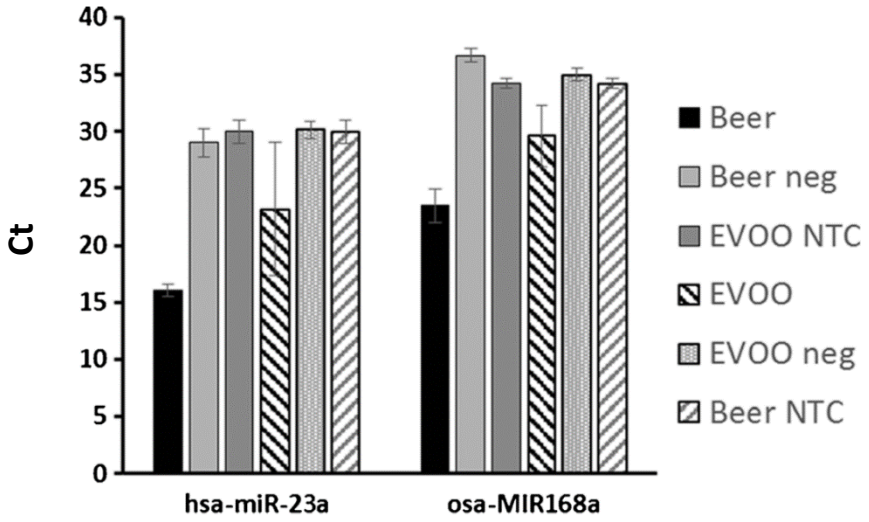

Figure 14: Ct values of hsa-miR-23a and osa-MIR168a spike-in controls measured by RT-qPCR. 200 ng of both, hsa-miR-23a mimic and osa-miR168a sequence were added to the EVOO and beer samples before microRNA extraction. Both microRNAs were measured by RT-qPCR and the Ct values are shown. Beer and EVOO negative samples (neg) refers to samples without the spike-ins.NTC, Non-template control. Data represent mean ± SEM

1.2. Presence of exogenous microRNAs in human plasma samples

Although we did not confidently detect microRNAs in beer and EVOO, we searched for the presence of plant microRNAs in plasma samples after the ingestion of 40 mL of EVOO. 40 mL is the usual daily EVOO intake within the frame of the Mediterranean diet and the PREDIMED study showed that this amount is able to reduce cardiovascular mortality (72). All volunteers usually follow a diet close to the Mediterranean pattern and consume EVOO as the usual source of fat in their diet. Thus, the basal amount of EVOO microRNAs in their plasma was expected to be

high enough to be detected by NGS. However, we followed additional strategies to further increase the amount of EVOO microRNAs present in plasma and maximize their detection using NGS. First, our human study followed a postprandial charge design (microRNAs measured 2 h post-ingestion of 40 mL of EVOO). Second, we measured microRNAs in a concentrated pool of plasma from all volunteers.

Bioanalyzer results showed that 67 % of the sample corresponded to microRNAs (372.23 ng/µl) **(figure 14)**. NGS returned 2,178,271 reads of 8–76 nt, 81.46 % of them corresponded to reads of <20 nt, 89.79 % of them were duplicated. After filtering and aligning against *Viridiplantae* clade allowing a maximum of three mismatches (269), we obtained six different sequences that could be identified as plant microRNAs (Table 3). We only obtained one sequence that could be aligned with *Viridiplanae* microRNAs with 0 mismatches: vvi-miR3623-3p (grape). However only one read was detected. When we repeated the analysis allowing one mismatch, we did not identified any additional plant microRNA. However, we identified three microRNAs (*Medicago trunculata*, grape and *Brachypodium distachyon*), but with only 1–2 reads. When we allowed three mismatches, we identified two other microRNAs from grape.

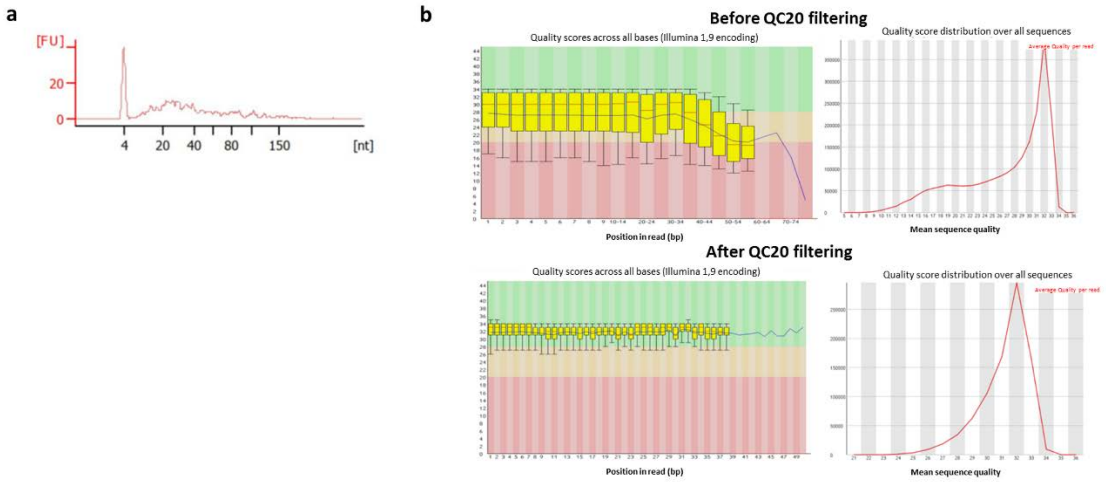

Figure 14: Electropherogram and Quality Control analyses of the NGS of 200 µl of plasma. miRCury Extraction Kit specific for biofluids was used to extract microRNAs from 200 µL of plasma. a. Bioanalyzer electropherogram. b. Upper panel shows the average QC value of the sequences before quality filtering. Lower panel shows the average QC value of the sequences after filtering by a threshold of 20.

Specifically, we detected 109,296 reads mapping to the gmamiR1529 sequence, 1088 of them aligned with ≥19 nucleotides. However, this finding should be cautiously interpreted because sensibility score of this alignment was low, it presented three mismatches and this sequence also corresponded to the endogenous human miR-302a/b-3p.Moreover, all the read sequences presented missing nucleotides when comparing with the canonical sequence **(table 9)**.

Table 9: *Viridiplantae* microRNA sequences identified in plasma samples after consumption of 40 mL of EVOO

Sequence ID	miRNA	Read sequence	Mistmatches	Reads	Specie
3MFN1:02913:01878	Read sequence	TGGTGCTTGGACGAATTTGCT	0	1	grape
	vvi-miR3623-3p	TGGTGCTTGGACGAATTTGCTa			
3MFN1:03137:01810	Read sequence	TTAAGGTGATTATTGCGGC	2	1	*Medicago trunculata*
	mtr-miR2590b	aatcTaAAGGTGATTATTGtGCc			
2DHGC:00857:00054	Read sequence	GTAGTCGATGGGAAATTG	2	1	grape
	vvi-miR3626-5p	gGTAGTCGcTGtGAAATTGaa			
3MFN1:02730:01211	Read sequence	TCGAGTCGGCCTGCGCGG	2	2	*Brachypodium distachyon*
	bdi-miR7778-5p	gagcaTCGtGTCGGCgTGCGCGGc			
2DHGC:02083:01146	Read sequence	TTAATGGAAACACTTAATCTT	3	8	soybean
	gma-miR1529	TTAAtGGAAACAaTTAATCgTta			
3MFN1:01049:00156	Read sequence	TTCATGGAAACACTTAATC	3	109,296	soybean
	gma-miR1529	TTaAaGGAAACAaTTAATCgtta			

2. VOHF Study

2.1. Postpandrial modification of plasma microRNA levels with functional olive oils

Four microRNAs were significantly modulated by L-EVOO (**figure 15**). Let-7e showed a bimodal distribution with two peaks of repression at 1 and 4 hours. miR-17 and miR-20a were upregulated after consumption of L-EVOO along the whole postpandrial curve with a maximum at 4 and 1 hours, respectively. On the other hand, miR-328 was downregulated after intake of L-EVOO with a minimum of expression at 1h.

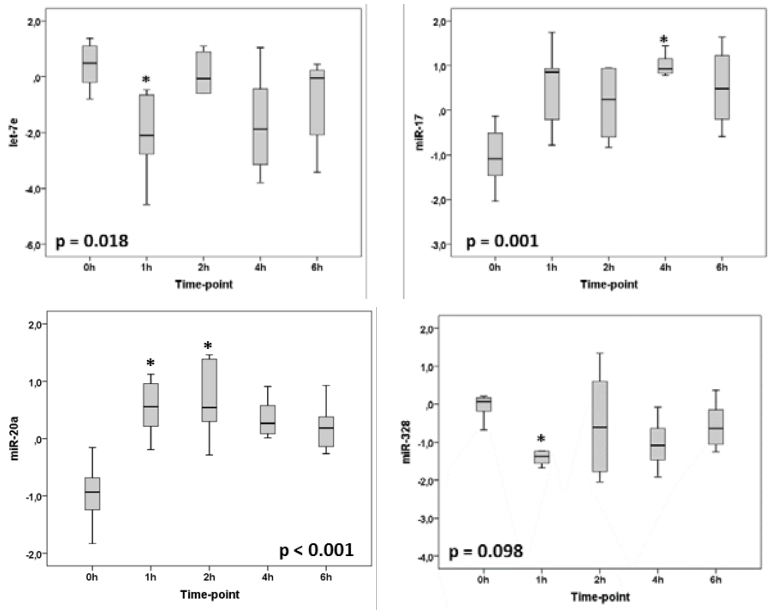

Figure 15. microRNAs modulated by L-EVOO. Box plots showing Log_2 transformed relative quantification levels of indicated microRNAs along the postprandial phase. Plasma microRNA levels were calculated with the $2^{-\Delta\Delta Ct}$ method comparing with 0h time-point. P value refers to the intra-subjects comparison of the paired-repeated measures ANOVA with Bonferroni correction. * p<0.05. Data represent mean ± SEM

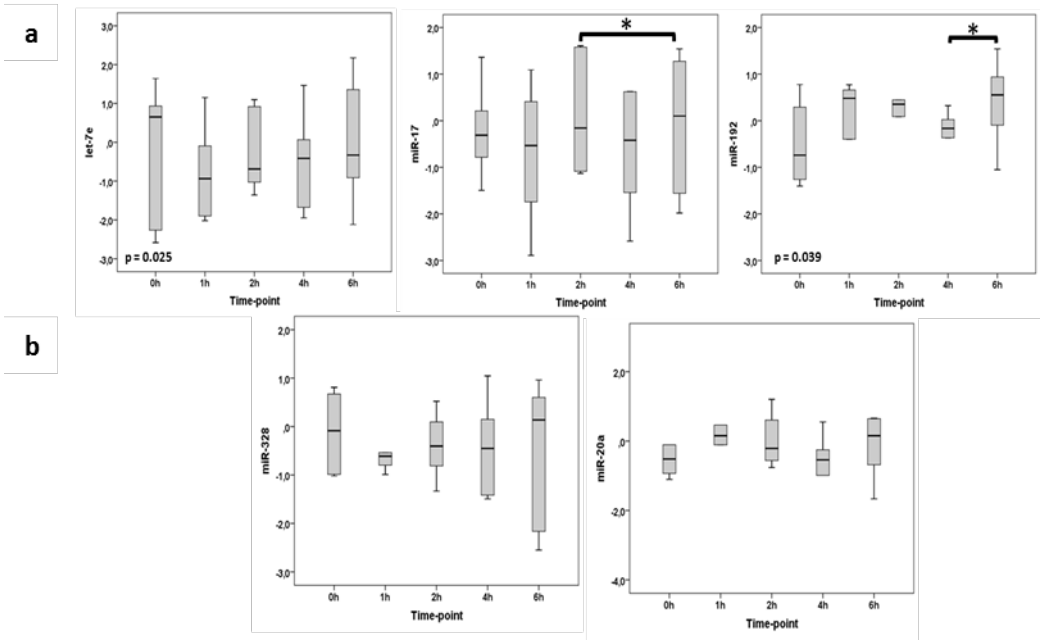

Figure 16. microRNAs modulated by M-EVOO. a) Box plots showing Log_2 transformed relative quantification levels of let-7e, miR-17 and miR-192 along the postpandrial phase. Plasma microRNA levels were calculated with the $2^{-\Delta\Delta Ct}$ method comparing with 0h time-point. P value refers to the intra-subjects comparison of the paired-repeated measures ANOVA with Bonferroni correction. * $p<0.05$. b) Box plots showing Log_2 transformed relative quantification levels of miR-328 and miR-20a along the postpandrial phase. Plasma microRNA levels were calculated with the $2^{-\Delta\Delta Ct}$ method comparing with 0h time-point. Data represent mean ± SEM

Three microRNAs were modulated by M-EVOO **(figure 16A)**. Let-7e showed a similar modification pattern than with L-EVOO (p = 0.025), although these results were not significant after Bonferroni correction in intra-subjects comparisons. miR-17 linear trend was not significantly modified, although we observed a significant overexpression at 6h when comparing with 2 hours. Finally, miR-192 was significantly upregulated by the M-EVOO. We observed that miR-328 and miR-20a showed the same trend than with L-EVOO although this trend did not reach statistical significance (f**igure 16B)**.

H-EVOO modified significantly the expression of 4 microRNAs (f**igure 17**). All of them were downregulated after 2 hours of the H-EVOO ingestion. The expression of let-7e increased up to basal levels at 4 hours, whereas the expression of the others remained downregulated.

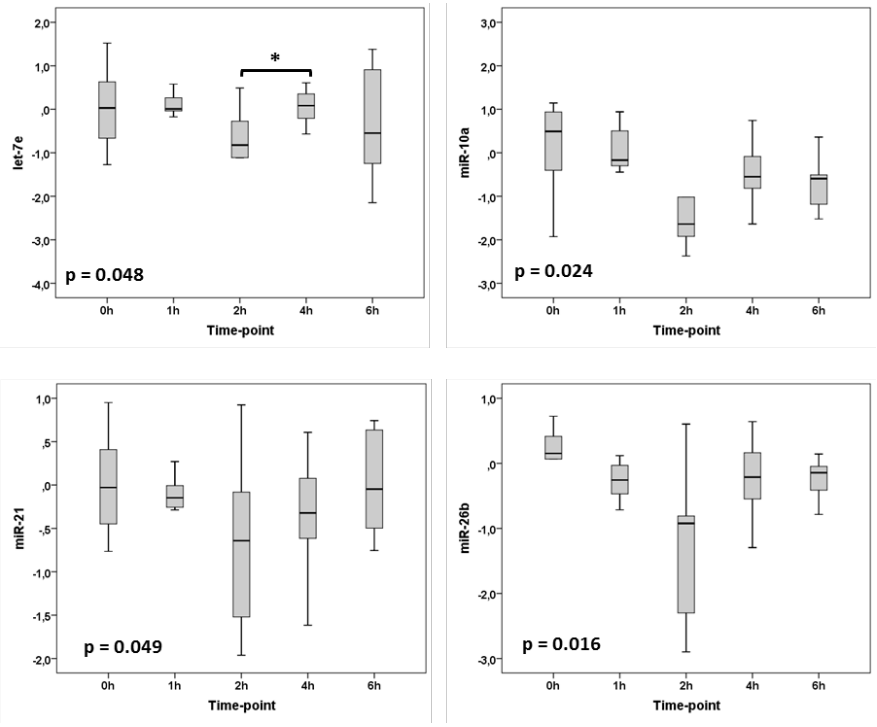

Figure 17. microRNAs modulated by H-EVOO. A. Box plots showing Log_2 transformed relative quantification levels of indicated microRNAs along the postpandrial phase. Plasma microRNA levels were calculated with the $2^{-\Delta\Delta Ct}$ method comparing with 0h time-point. P value refers to the intra-subjects comparison of the Paired-repeated measures ANOVA with Bonferroni correction. * $p<0.05$. Data represent mean ± SEM

2.2. In silico functional analyses

We performed a functional analysis of the microRNAs differentially expressed in order to define the pathways and biological processes that they regulate. For that purpose, we used miRWalk database to select the genes that are predicted or validated targets of at least 2/4 microRNAs in L-EVOO, 2/3 microRNAs in M-EVOO and 2/4 microRNAs in H-EVOO.

Functional analyses showed that 1361 genes were targets of microRNAs modulated by L-EVOO **(figure 18a)**. between them 1070 genes are common for miR-17 and miR20a, that belongs to the same cluster. Targeted genes were mainly related to different types of cancer and related processes (p-53 signalling and cell cycle progression) (**table 18b**). But among the enriched pathways, we also observed MAPK signalling, considered an aging pathway involved in the regulation of the cellular energetic state and nutrient metabolism.

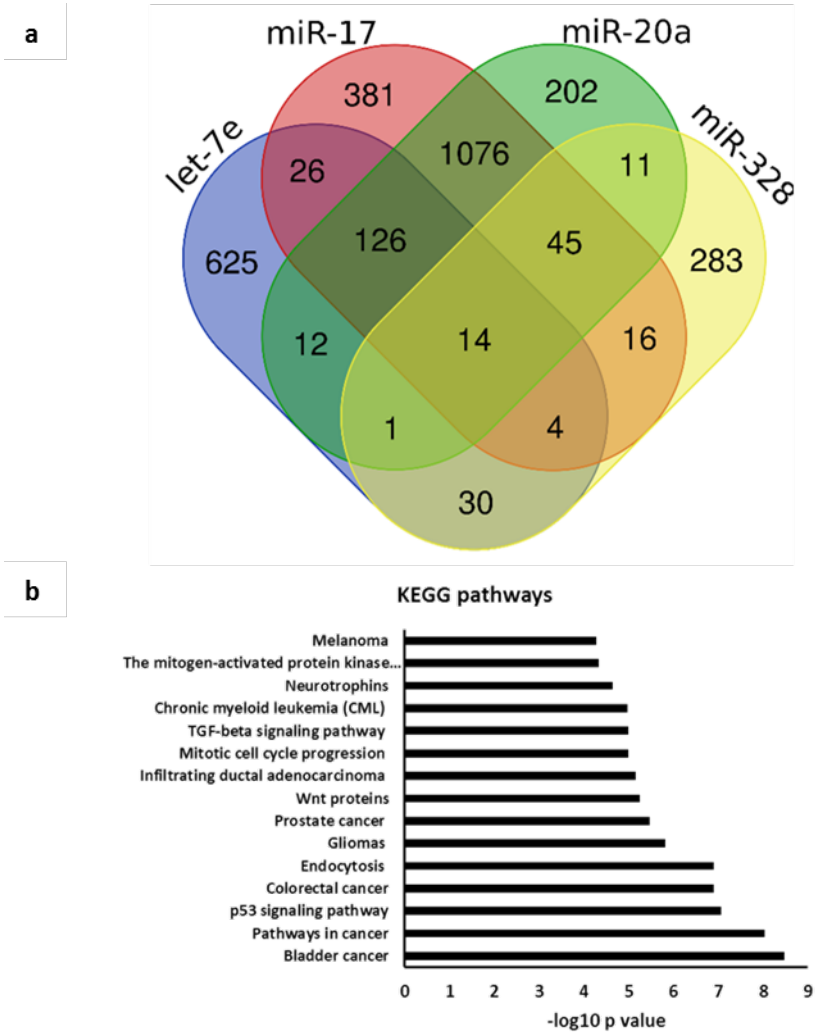

Figure 18. Functional analysis of microRNAs modulated by L-EVOO. a) Venn Diagram showing common targets of the modulated microRNAs and b) Bar chart of the top 15 most represented KEGG pathways (-$\log_{10}$ p value adjusted by FDR) for L-EVOO.

Fourteen genes were predicted or validated targets of all those microRNAs (**figure 18a)**. Among them, we found *PRKCB* (protein kinase C, beta subunit), involved in endothelial cell proliferation, intestinal sugar absorption, insulin signalling and B-cell activation (**figure 18b and table 9**) (270)**.** We also found *CRY2* (cryptochrome 2), with a well-known role in the circadian system, and *SCD* (stearoyl CoA desaturase), involved in monounsaturated fatty acid synthesis. Interestingly, 30 genes were predicted or validated targets of let-7e and miR-328, both downregulated at 1h **(figure 18a and table 9).**

381 genes were validated and predicted targets of microRNAs modulated by M-EVOO **(figure 19a)**. They were involved in different types of cancer and related processes (DNA replication, cell cycle progression and apoptosis) (**table 10**). Interestingly, those genes were also involved in insulin signalling (**figure 19b**). 25 target genes were predicted targets for all three microRNAs. Among them, we found *PRKAA2* (*protein kinase AMP-activated catalytic subunit alpha 2*), the catalytic subunit of the AMPK cellular energetic sensor **(figure 19b, and table 10).**

Table 9: Genes commonly targeted by, at least, two microRNAs modified by L-EVOO

Gene symbol	Gene name	GO Biological Process
let-7e + miR-17 + miR-20a + miR-38		
IPO9	Importin 9	binding and protein transporter activity
PRKCB	Protein Kinase C Beta	transferase activity, transferring phosphorus-containing groups and protein tyrosine kinase activity.
ANKRD52	Ankyrin Repeat Domain 52	protein binding
RAB11FIP4	RAB11 Family Interacting Protein 4	calcium ion binding and Rab GTPase binding
RAB22A	Member RAS Oncogene Family	GTP binding and GDP binding.
HMGB1	High Mobility Group Box 1	chromatin binding
CRY2	Cryptochrome Circadian Regulator 2	protein kinase binding and kinase binding.
USP24	Ubiquitin Specific Peptidase 24	binding and cysteine-type peptidase activity.
SCD	Stearoyl-CoA Desaturase	iron ion binding and stearoyl-CoA 9-desaturase activity
TXLNA	Taxilin Alpha	cytokine activity and high molecular weight B cell growth factor receptor binding
AFF2	AF4/FMR2 Family Member 2	G-quadruplex RNA binding.
ZNF280B	Zinc Finger Protein 280B	DNA and metal ion binding
HIF1AN	Hypoxia Inducible Factor 1 Alpha Subunit Inhibitor	protein homodimerization activity and oxidoreductase activity
CRK	CRK Proto-Oncogene, Adaptor Protein	tein domain specific binding and SH3/SH2 adaptor activity
let-7e + miR-328		
UNC5A	Unc-5 Netrin Receptor A	plasma membrane
MED13L	Mediator Complex Subunit 13 Like	RNA polymerase II transcription cofactor activity
SEC14L5	SEC14 Like Lipid Binding 5	transporter activity
KATNB1	Katanin Regulatory Subunit B1	protein heterodimerization activity
SLCO2A1	Solute Carrier Organic Anion Transporter Family Member 2A1	transporter activity and prostaglandin transmembrane transporter activity
YWHAQ	Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Theta	protein domain specific binding and protein N-terminus binding
AHCYL2	Adenosylhomocysteinase Like 2	NAD binding and adenosylhomocysteinase activity
STK40	Serine/Threonine Kinase 40	transferase activity, transferring phosphorus-containing groupsand protein tyrosine kinase activity
RUNX1T1	RUNX1 Translocation Partner 1	DNA binding transcription factor activity and identical protein binding
DTX4	Deltex E3 Ubiquitin Ligase 4	ligase activity
PLXNA4	Plexin A4	receptor activity and semaphorin receptor activity
IQGAP3	IQ Motif Containing GTPase Activating Protein 3	calmodulin binding and Ras GTPase binding.
UROC1	Urocanate Hydratase 1	urocanate hydratase activity
EN2	Engrailed Homeobox 2	sequence-specific DNA binding
SEMA3F	Semaphorin 3F	receptor activity and chemorepellent activity.
ATXN2L	Ataxin 2 Like	RNA Binding
STARD7	StAR Related Lipid Transfer Domain Containing 7	lipid binding
CHST3	Carbohydrate Sulfotransferase 3	sulfotransferase activity and chondroitin 6-sulfotransferase activity
ZNF609	Zinc Finger Protein 609	nucleic acid and metal ion binding
AEBP2	AE Binding Protein 2	RNA polymerase II proximal promoter sequence-specific DNA binding and transcriptional repressor activity, RNA polymerase II proximal promoter sequencespecific DNA binding.
PAPPA	Pappalysin 1	metalloendopeptidase activity and endopeptidase activity
SMC1A	Structural Maintenance Of Chromosomes 1A	chromatin binding
ZCCHC24	Zinc Finger CCHC-Type Containing 24	nucleic acid binding.
SLC38A2	Solute Carrier Family 38 Member 2	symporter activity and amino acid transmembrane transporter activity
ARHGAP19	Rho GTPase Activating Protein 19	GTPase activator activity
DIAPH1	Diaphanous Related Formin 1	nucleotide binding
IGF1R	Insulin Like Growth Factor 1 Receptor	identical protein binding and protein kinase activity
SNX30	Sorting Nexin Family Member 30	phosphatidylinositol binding
OTUD5	OTU Deubiquitinase 5	thiol-dependent ubiquitin-specific protease activity
SERBP1	SERPINE1 MRNA Binding Protein 1	mRNA 3-UTR binding

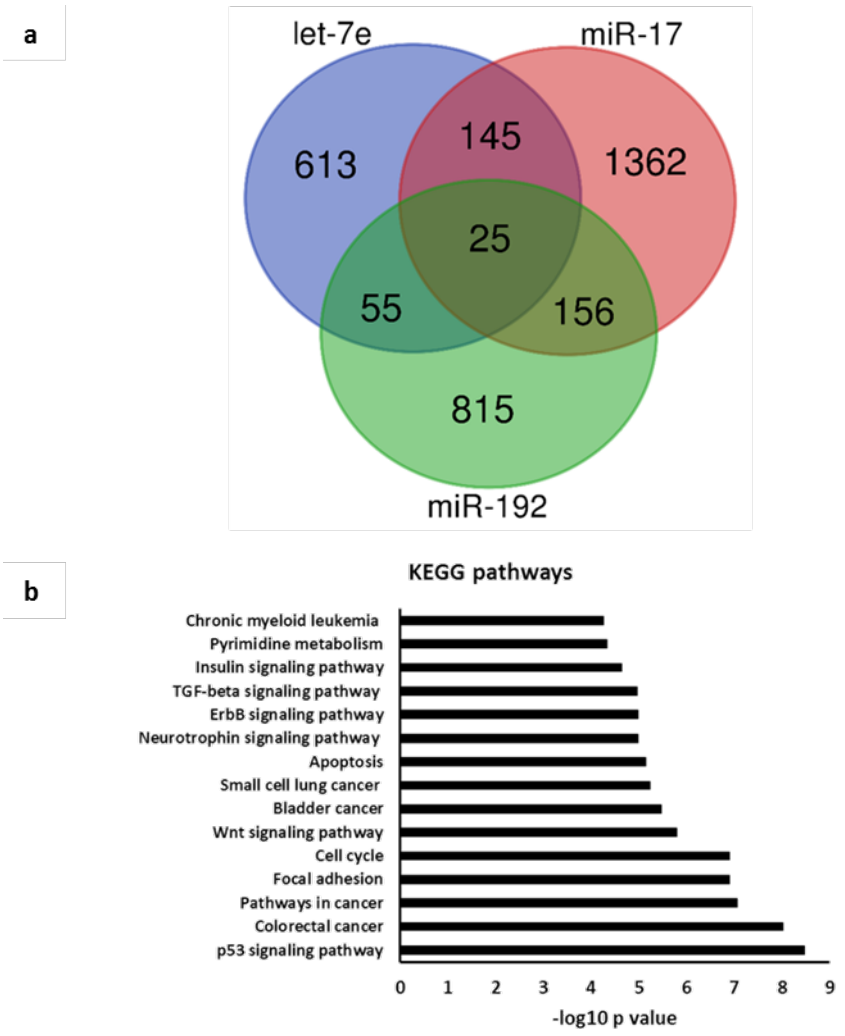

Figure 19. Functional analysis of microRNAs modulated by M-EVOO: a) Venn Diagram showing common targets of the modulated microRNAs and b) Bar chart of the top 15 most represented KEGG pathways (-$\log_{10}$ p value adjusted by FDR) for M-EVOO.

Table 10: Genes commonly targeted by all the 3 microRNAs modulated by M-EVOO.

Gene symbol	Gene name	GO Biological Process
NHLRC3	NHL Repeat Containing 3	extracellular exosome
RACGAP1	Rac GTPase Activating Protein 1	protein kinase binding and microtubule binding
NCOA3	Nuclear Receptor Coactivator 3	chromatin binding and transcription coactivator activity
XIAP	X-Linked Inhibitor Of Apoptosis	ligase activity and ubiquitin protein ligase activity
SLC30A7	Solute Carrier Family 30 Member 7	cation transmembrane transporter activity
NPHP3	Nephrocystin 3	protein binding
TMEM167A	Transmembrane Protein 167A	Golgi apparatus
SGCD	Sarcoglycan Delta	cytoplasm
MKI67	Marker Of Proliferation Ki-67	protein C-terminus binding
DNAL1	Dynein Axonemal Light Chain 1	motor activity
EEA1	Early Endosome Antigen 1	protein homodimerization activity and 1-phosphatidylinositol binding
ZNF652	Zinc Finger Protein 652	nucleic acid binding
DCBLD2	Discoidin, CUB And LCCL Domain Containing 2	protein binding
KATNAL1	Katanin Catalytic Subunit A1 Like 1	microtubule binding and microtubule-severing ATPase activity
SCD	Stearoyl-CoA Desaturase	iron ion binding and stearoyl-CoA 9-desaturase activity
GABPB1	GA Binding Protein Transcription Factor Beta Subunit 1	DNA binding transcription factor activity and transcription regulatory region DNA binding
POLQ	DNA Polymerase Theta	nucleic acid binding and damaged DNA binding
MAP3K1	Mitogen-Activated Protein Kinase Kinase Kinase 1	transferase activity, transferring phosphorus-containing groups and protein tyrosine kinase activity
PRKAA2	Protein Kinase AMP-Activated Catalytic Subunit Alpha 2	transferase activity, transferring phosphorus-containing groups and protein tyrosine kinase activity
SLC1A4	Solute Carrier Family 1 Member 4	chloride channel activityand L-serine transmembrane transporter activity
PLAGL2	PLAG1 Like Zinc Finger 2	RNA binding transcription factor activity and transcriptional activator activity, RNA polymerase II proximal promoter sequence-specific DNA binding
NIPA1	Non Imprinted In Prader-Willi/Angelman Syndrome 1	magnesium ion transmembrane transporter activity
CRK	CRK Proto-Oncogene, Adaptor Protein	protein domain specific binding and SH3/SH2 adaptor activity
UBE2V2	Ubiquitin Conjugating Enzyme E2 V2	ubiquitin protein ligase binding and acid-amino acid ligase activity
MDM4	MDM4, P53 Regulator	enzyme binding

454 genes were validated and predicted targets of microRNAs modulated by H-EVOO (**figure 20a)**. They were involved in different types of cancer and related pathways (p53 signalling, focal adhesion), but they were also associated with PIK3/AKT signalling (**figure 20b**). *YOD1* gene was the only predicted target of all four microRNAs. *YOD1* is a protein deubiquitinase involved in many cellular processes including cell cycle, signal transduction and inflammation (271).

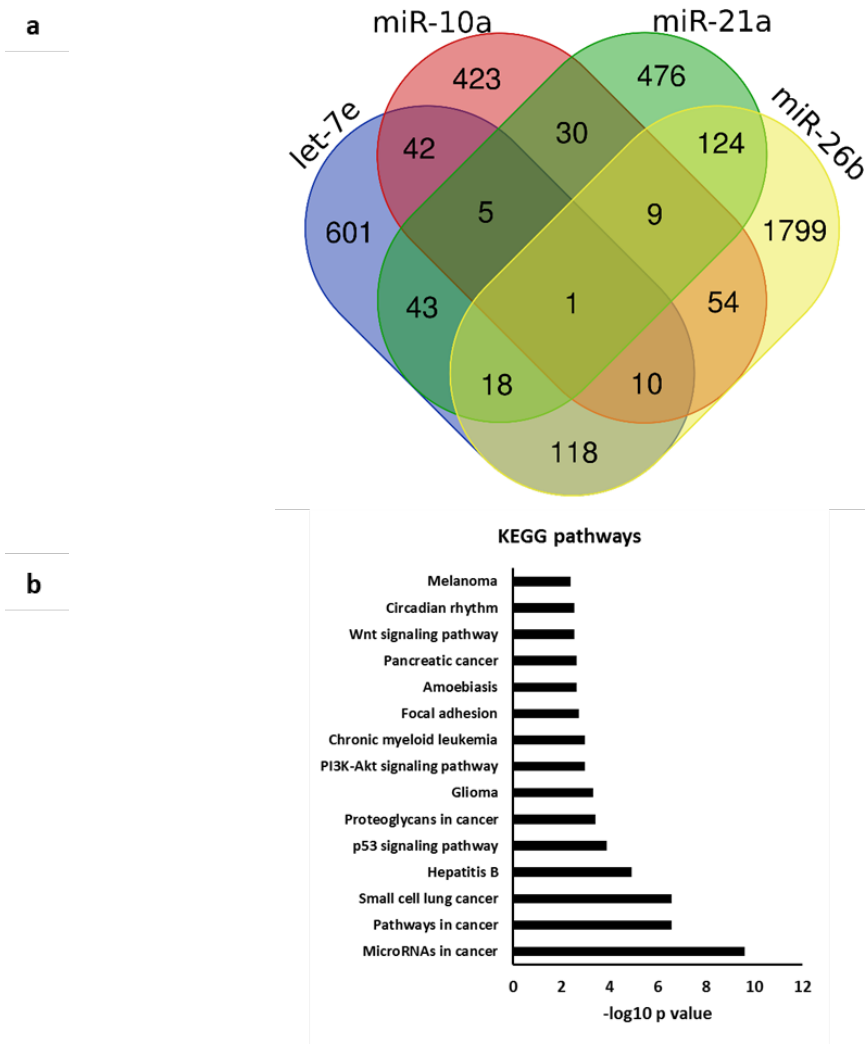

Figure 20. Functional analysis of microRNAs modulated by H-EVOO. a) Venn Diagram showing common targets of the modulated microRNAs and b) Bar chart of the top 15 most represented KEGG pathways (-$\log_{10}$ p value adjusted by FDR).for H-EVOO.

2.3. MicroRNA levels according to type of functional oil

Then, we performed interaction analyses to compare microRNA levels across functional olive oils **(figure 21a)**. Interestingly, let-7e showed the same bimodal behaviour in all the functional oils, although the trend seems to be delayed 1 hour with the H-EVOO. At 1 hour, plasma let-7e levels were 4.608-fold downregulated after ingestion of L-EVOO, compared with the levels at 1h after ingestion of H-EVOO ($p<0.001$). At 4 hours, plasma levels of let-7e after ingestion of L-EVOO were 3.364-fold downregulated when comparing with levels after ingestion of H-EVOO ($p = 0.032$). Interaction analyses for miR-126, miR-24 and miR-328 levels were not significant.

However, it is worth mentioning that circulating levels with L-EVOO and M-EVOO showed a more similar trend between them than with H-EVOO. When we performed pair comparisons, we observed that miR-126 plasma levels after 4 hours of the ingestion of M-EVOO were 1.541-fold downregulated as compared with the ingestion of H-EVOO at the same time ($p=0.047$). miR-24 levels 1 hour after the ingestion of L-EVOO were 1.862-fold lower than after the ingestion of H-

EVOO at the same time-point (p=0.016), whereas miR-328 levels 1 hour after the ingestion of L-EVOO were 2.604-fold lower than after the ingestion of H-EVOO at the same time-point (p=0.017). Interaction between circulating miR-26b levels and the type of functional oil was significant (p=0.043) with a more similar dynamics between low and medium EVOO than with H-EVOO. More specifically, miR-26 circulating levels barely changed along the postpandrial phase with low and medium EVOO, while they are significantly downregulated after 2 hours of the ingestion of H-EVOO. The positive fold change of miR-26b levels after 2 hours of the ingestion of L-EVOO and M-EVOO as compared with H-EVOO was 2.369 (p=0.031) and 2.32 (p=0.051), respectively. Circulating levels of miR-192 were modified by M-EVOO and H-EVOO in an opposite manner. While M-EVOO lead to a postpandrial increase, H-EVOO resulted in a postpandrial decrease of circulating miR-192 (FC (2h) =2.025; p=0.041). Finally, miR-20a plasma levels were similarly modified by L-EVOO and M-EVOO. However, the early increased observed was more pronounce with L-EVOO, while the late decrease was more pronounce with M-EVOO. After 6 hours, levels had come back to basal levels in the two cases.

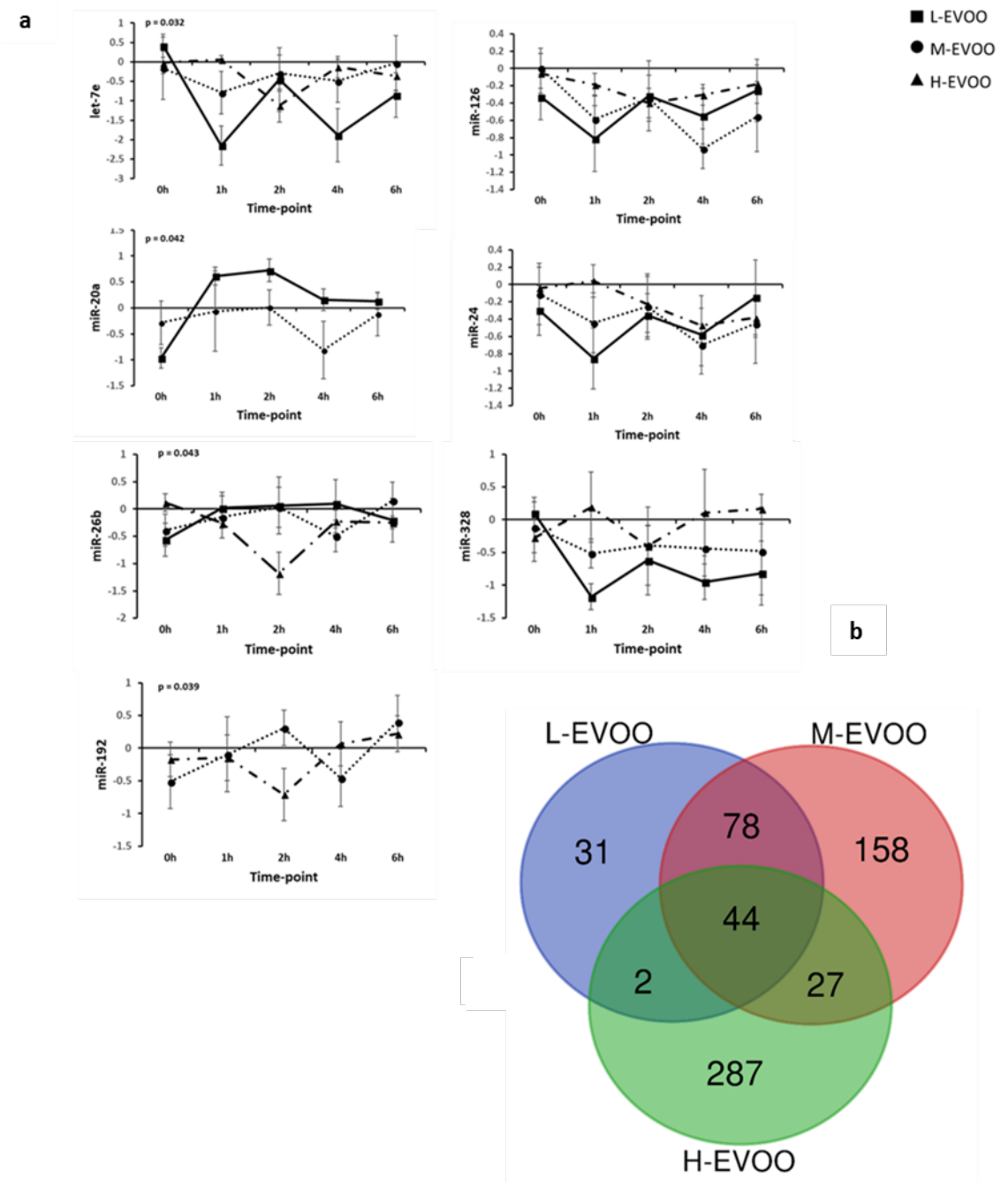

Figure 21: Differentially modulated microRNAs according to the type of functional oil. a) Interaction plots showing Log_2 transformed relative quantification levels of indicated microRNAs along the postpandrial phase in the three functional olive oils. Plasma microRNA levels were calculated with the $2^{-\Delta\Delta Ct}$ method comparing with 0h time-point. P value refers to analyses of the paired-repeated measures ANOVA with Bonferroni correction with oil type as interaction parameter. * $p<0.05$, ** $p<0.001$. b) Venn Diagram showing common targets of the modulated microRNAs in the three functional oils.

We then performed an *in silico* analysis to define which genes are common targets of microRNAs modulated be the three functional oils **(figure 21b)**. 44 genes were predicted and validated targets of microRNAs modified by all three oils. Those genes were involved in different types of cancer as well as in AMPK, MAPK and FOXO signalling and the circadian system **(figure 22 and table 11)**. Only two genes, *NUFIP2* (FMR1 interacting protein 2) and *ABCG2* (ATP binding cassette

subfamily G member 2), were common targets among microRNAs modified by L-EVOO and H-EVOO. *NUFIP2* is a RNA binding protein that regulates RNA degradation (272) and whose intracellular location is cell cycle-dependent. ABCG2 is an ABC xenobiotic transporter with a role in multi-drug resistance (273). In addition to this, *CCND1, STAT3, NCOA3, MYC, SIAP* and *TNFRSF108* conform an interaction core of genes involved in Jak-STAT signaling pathway, immunity, cell division, cell death and tumour formation (274).

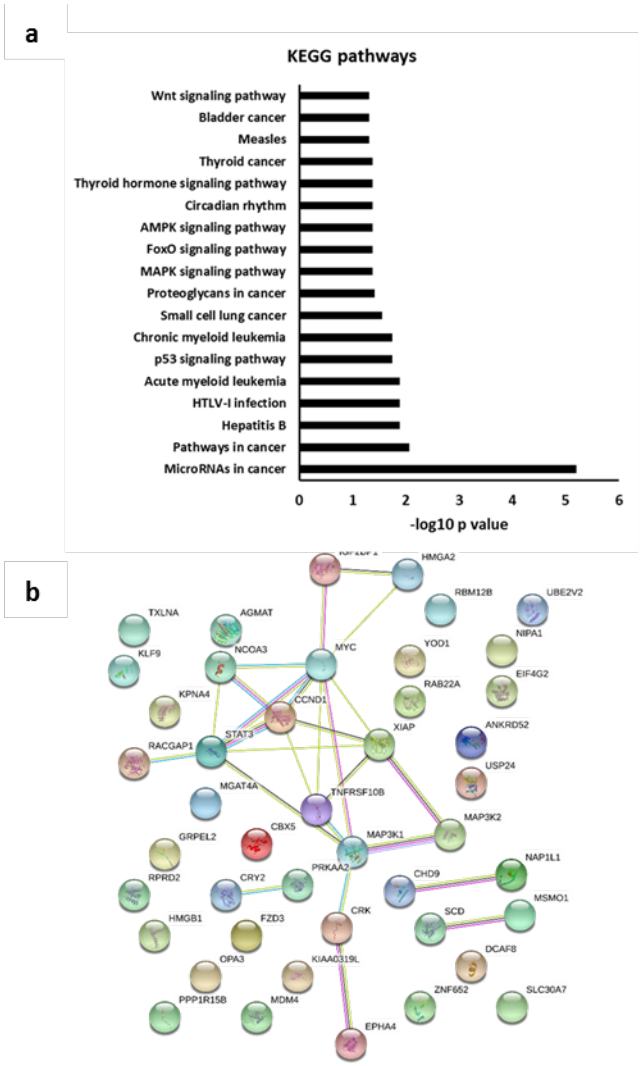

Figure 21: Biocomputational analyses of predicted and validated gene targets of microRNAs modulated by all three functional oils. A. Bar chart of the top 15 most represented KEGG pathways ($-\log_{10}$ p value adjusted by FDR). B. Protein-protein interaction network of genes targeted by microRNAs modulated by all three functional oils.

Table 11: Genes targeted by microRNAs common to all the three functional oils

Gene symbol	Gene name	GO Biological Process
PRKAA2	Protein Kinase AMP-Activated Catalytic Subunit Alpha 2	transferase activity, transferring phosphorus-containing groups and protein tyrosine kinase activity
KPNA4	Karyopherin Subunit Alpha 4	binding and protein transporter activity
CHD9	Chromodomain Helicase DNA Binding Protein 9	nucleic acid binding and helicase activity
CRY2	Cryptochrome Circadian Regulator 2	protein kinase binding and kinase binding.
PPP1R15B	Protein Phosphatase 1 Regulatory Subunit 15B	protein serine/threonine phosphatase activity
YOD1	YOD1 Deubiquitinase	thiol-dependent ubiquitin-specific protease activity and Lys48-specific deubiquitinase activity
MAP3K2	Mitogen-Activated Protein Kinase 2	transferase activity, transferring phosphorus-containing groups and protein tyrosine kinase activity
USP24	Ubiquitin Specific Peptidase 24	binding and cysteine-type peptidase activity
RACGAP1	Rac GTPase Activating Protein 1	protein kinase binding and microtubule binding
TNFRSF10B	TNF Receptor Superfamily Member 10b	receptor activity and TRAIL binding
EPHA4	EPH Receptor A4	identical protein binding and protein kinase activity
RAB22A	Member RAS Oncogene Family 22A	GTP binding and GDP binding
MGAT4A	Mannosyl (Alpha-1,3-)-Glycoprotein Beta-1,4-N-Acetylglucosaminyltransferase, Isozyme A	transferase activity, transferring hexosyl groups and alpha-1,3-mannosylglycoprotein 4-beta-N-acetylglucosaminyltransferase activity
MSMO1	Methylsterol Monooxygenase 1	oxidoreductase activity and C-4 methylsterol oxidase activity
ANKRD52	Ankyrin Repeat Domain 52	protein binding
OPA3	Outer Mitochondrial Membrane Lipid Metabolism Regulator 3	mitochondrion
NIPA1	Non Imprinted In Prader-Willi/Angelman Syndrome 1	magnesium ion transmembrane transporter activity
NCOA3	Nuclear Receptor Coactivator 3	chromatin binding and transcription coactivator activity
CRK	CRK Proto-Oncogene, Adaptor Protein	protein domain specific binding and SH3/SH2 adaptor activity
SCD	Stearoyl-CoA Desaturase	iron ion binding and stearoyl-CoA 9-desaturase activity
STAT3	Signal Transducer And Activator Of Transcription 3	DNA binding transcription factor activity and sequence-specific DNA binding
XIAP	X-Linked Inhibitor Of Apoptosis	ligase activity and ubiquitin protein ligase activity
FZD3	Frizzled Class Receptor 3	G-protein coupled receptor activity and PDZ domain binding
KIAA0319L	KIAA0319 Like	protein binding
GRPEL2	GrpE Like 2, Mitochondrial	protein homodimerization activity and chaperone binding
CCND1	Cyclin D1	protein kinase activity and enzyme binding
SLC30A7	Solute Carrier Family 30 Member 7	cation transmembrane transporter activity
CBX5	Chromobox 5	protein homodimerization activity and histone deacetylase binding
UBE2V2	Ubiquitin Conjugating Enzyme E2 V2	ubiquitin protein ligase binding and acid-amino acid ligase activity
EIF4G2	Eukaryotic Translation Initiation Factor 4 Gamma 2	binding
RBM12B	RNA Binding Motif Protein 12B	RNA and protein binding
MYC	MYC Proto-Oncogene, BHLH Transcription Factor	DNA binding transcription factor activity and RNA polymerase II proximal promoter sequence-specific DNA binding
AGMAT	Agmatinase	agmatinase activity
KLF9	Kruppel Like Factor 9	DNA binding transcription factor activity
TXLNA	Taxilin Alpha	cytokine activity and high molecular weight B cell growth factor receptor binding
MAP3K1	Mitogen-Activated Protein Kinase Kinase Kinase 1	transferase activity, transferring phosphorus-containing groups and protein tyrosine kinase activity
RPRD2	Regulation Of Nuclear Pre-MRNA Domain Containing 2	DNA-directed RNA polymerase II, holoenzyme
IGF2BP1	Insulin Like Growth Factor 2 MRNA Binding Protein 1	nucleic acid binding and RNA binding
HMGA2	High Mobility Group AT-Hook 2	enzyme binding and transcriptional activator activity, RNA polymerase II proximal promoter sequence-specific DNA binding
MDM4	MDM4, P53 Regulator	enzyme binding
HMGB1	High Mobility Group Box 1	chromatin binding
ZNF652	Zinc Finger Protein 652	nucleic acid binding
NAP1L1	Nucleosome Assembly Protein 1 Like 1	RNA and protein binding
DCAF8	DDB1 And CUL4 Associated Factor 8	protein binding

3. MiRoBEER study

3.1. Characteristics of the population

During the intervention none of the biochemistry or diet parameters were significantly modified except MUFA consumption, that decreased in every intervention period (p=0.02) **(table 12).** Interestingly, we can see a tendency to decrease cholesterol levels and PCR levels in every intervention period while we observed an increase of triglycerides in intervention periods. Regarding to diet changes, we observed a lower caloric intake, less carbohydrates, less fat and

saturated fatty acids (SFA) in every intervention point while the vegetal fiber increased, although these changes were not significant **(table 12)**

Table 12: Population description and biochemistry and diet parameters measured during the clinical trial. Values are expressed in mean ± SEM.

	WO1	Beer	WO2	Non-alcoholic beer	p value
Height (m)	1.70 ± 0.05	1.70 ± 0.05	1.70 ± 0.05	1.70 ± 0.05	n.s.
Weight (kg)	90.36 ± 12.46	90.57 ± 11.84	90.57 ± 11.84	90.69 ± 12.07	n.s.
BMI (kg/m2)	31.07 ± 2.6	31.13 ± 2.61	31.13 ± 2.61	31.03 ± 2.48	n.s.
Fat mass (%)	32.61 ± 3.33	32.56 ± 4.17	32.56 ± 4.17	32.64 ± 3.5	n.s.
Muscular mass (%)	30.87 ± 1.76	30.63 ± 2.20	30.63 ± 2.20	30.6 ± 1.7	n.s.
GEB (kcal)	1853.09 ± 177.88	1848.43 ± 169.57	1849.53 ± 171.45	1850.86 ± 170.17	n.s.
Visceral fat	15.86 ± 2.27	15.71 ± 1.98	15.8 ± 2	16 ± 2.16	n.s.
Cholesterol (mg/dl)	214.61 ± 43.21	203.56 ± 31.58	210.71 ± 34.99	205.94 ± 25.76	n.s.
HDL cholesterol (mg/dl)	49.19 ± 13.41	45.17 ± 11.71	56.73 ± 21.6	45.64 ±10.93	n.s.
LDL cholesterol (mg/dl)	134.63 ± 28.41	123.70 ±18.93	136.3 ± 24.86	130.38 ±14.27	n.s.
Triglycerides (mg/dl)	136.14 ± 108.2	187.43 ± 149.67	132.43 ± 68.88	169.86 ± 165.07	n.s.
Apolipoprotein B (mg/dl)	118.03 ± 28.83	112.29 ± 20.52	113.5 ± 21.5	117.83 ± 16.86	n.s.
C reactive protein (mg/dl)	0.28 ± 0.19	0.23 ± 0.14	0.23 ± 0.13	0.23 ± 0.16	n.s.
Energy (kcal)	2412.33 ± 450.68	2318.71 ± 419.30	2489.71 ± 406.32	2095.71 ± 383.15	n.s.
Protein (g/d)	130.18 ± 34.26	103.56 ± 20.88	118.21 ±25.65	94.34 ± 28.58	n.s.
Carbohydrates (g/d)	252.5 ± 49.81	248.43 ± 53.42	266.71 ± 61.89	250.29 ± 43.14	n.s.
Sugar (g/d)	112.6 ± 27.32	111.97 ± 36.34	121.83 ± 29.41	123.51 ± 19.78	n.s.
Starch (g/d)	109.67 ± 29.94	122.39 ± 29.19	120.86 ± 18.2	112.1 ± 32.38	n.s.
Vegetal fiber (g/d)	18.68 ± 5.95	20.14 ± 3.96	20.7 ± 7.55	20.51 ± 8.47	n.s.
Lipids (g/d)	91.63 ± 19.50	81.50 ± 27.32	96 ± 23.23	70.7 ± 18.71	n.s.
SFA (g/d)	34.85 ± 9.98	29.24 ± 14.03	34.47 ± 12	25.59 ± 7.27	n.s.
MUFA (g/d)	34.15 ± 7.19	26.33 ± 7.20	33.74 ± 1.39	22.01 ± 4.87	0.02
PUFA (g/d)	11.23 ± 0.86	13.24 ± 5.31	14.1 ± 1.93	10.27 ± 4.59	n.s.
WO1: wash-out 1 ; Beer: alcoholic beer ; WO2: wash-out 2 ; Non-alcoholic beer; BMI: Body mass index, GEB: basal metabolic rate; SFA: saturated fatty acid; MUFA: monoinsaturade fatty acid; PUFA: poliinsaturated fatty					

3.2. Beer polyphenols measurements

IX has been described as a sensitive and specific urine marker of beer consumption (275) In order to confirm that all volunteers carried out the intervention properly, we measured the levels of IX and 8-PG in plasma and urine samples of volunteers.

For this purpose, we first measured the levels of these 2 polyphenols in beer samples used in this study. We obtained that IX levels are 5 times higher in beer compared to non-alcoholic beer while levels of 8-PG are 2.5 times higher in non-alcoholic beer compared to regular beer **(figure 22).**

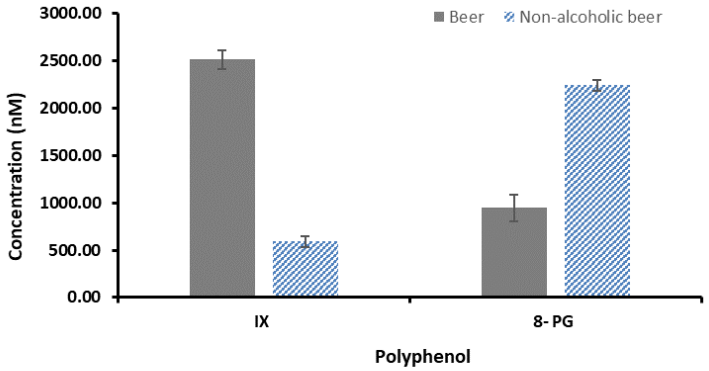

Figure 22: Measurement of beer polyphenols in beer and non-alcoholic beer. IX: Isoxanthohumol 8-PG: 8-Prenilnarigerin. Data represent mean ± SEM

After that, we analysed polyphenols levels in plasma and urine samples. Regarding to IX, we observed a non-significant tendency to increase IX levels in urine after intervention phases **(figure 23a)**. In plasma samples, we observed a significant increase of IX levels after beer ingestion (p=0.001), but not after non-alcoholic beer ingestion **(figure 23b)**.

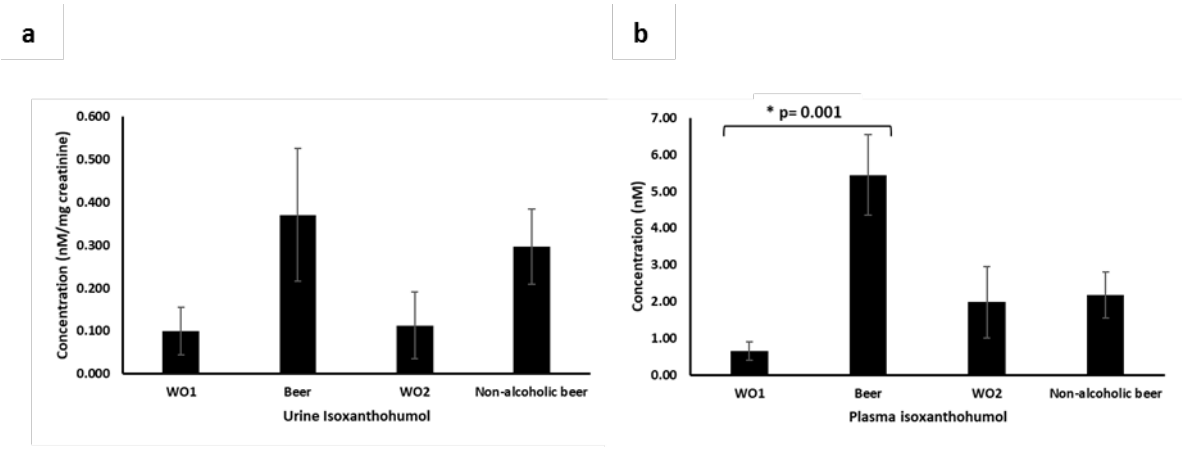

Figure 23: Measurement of IX in plasma and urine samples. p value refers to the intra-subjects comparison of the Paired-repeated measures T-student test. * p<0.05. IX: Isoxanthohumol, WO1: washout 1, Beer: alcoholic beer intervention, WO2: washout 2, non-alcoholic beer: non-alcoholic beer intervention. Data represent mean ± SEM

We did not observe an increase in urine or plasma 8-PG levels. On the contrary, we observed a non-significant decrease in urine 8-PG levels after beer consumption and a non-significant decrease in plasma after, both, beer and non-alcoholic beer consumption. **(figure 24 a and b)**. A reason for this variability could be that 8-PG is a secondary metabolite of IX with a great intra-subject variability in the metabolization of this compound.

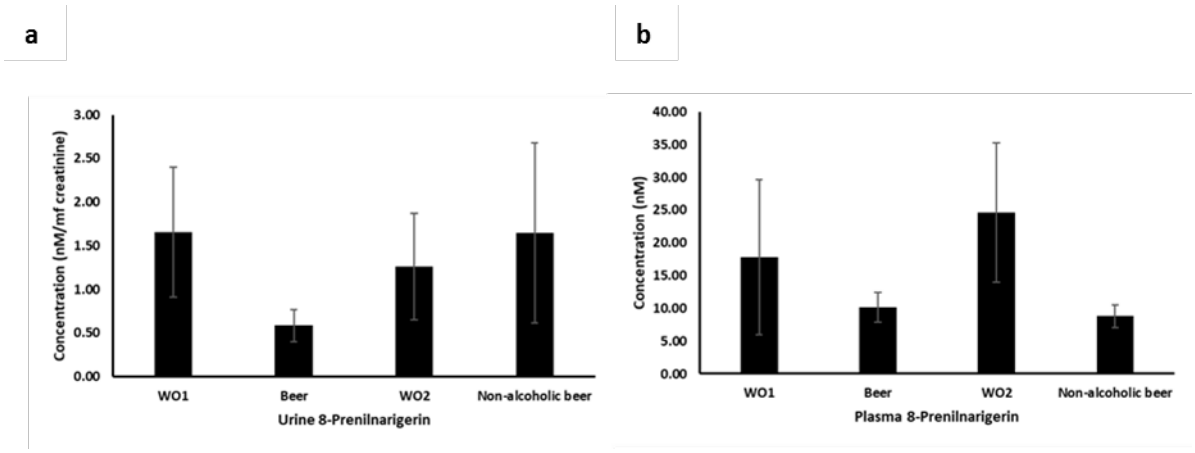

Figure 24: Measurement of 8-PG in urine (a) and plasma (b) samples. 8-PG: 8- Prenylnaringenin, WO1: washout 1, Beer: alcoholic beer intervention, WO2: washout 2, non-alcoholic beer: non-alcoholic beer intervention. Data represent mean ± SEM

3.3. Post-intervention modification of plasma microRNA levels with alcoholic and non-alcoholic beer intake.

4 circulating microRNAs were significantly modulated by beer intake **(figure 25)**. miR-155 levels increase after beer ingestion and decrease after non-alcoholic beer ingestion. Although these changes were not statistically significant, we observed that levels of miR-155 were significantly lower after non-alcoholic beer intake comparing to alcoholic beer intake (p= 0.036). A similar response was observed for miR-328 (p= 0.036) and miR-92a (p= 0.039). Finally, miR-320 was repressed after both interventions, but only non-alcoholic beer intervention was significant (p=0.022).

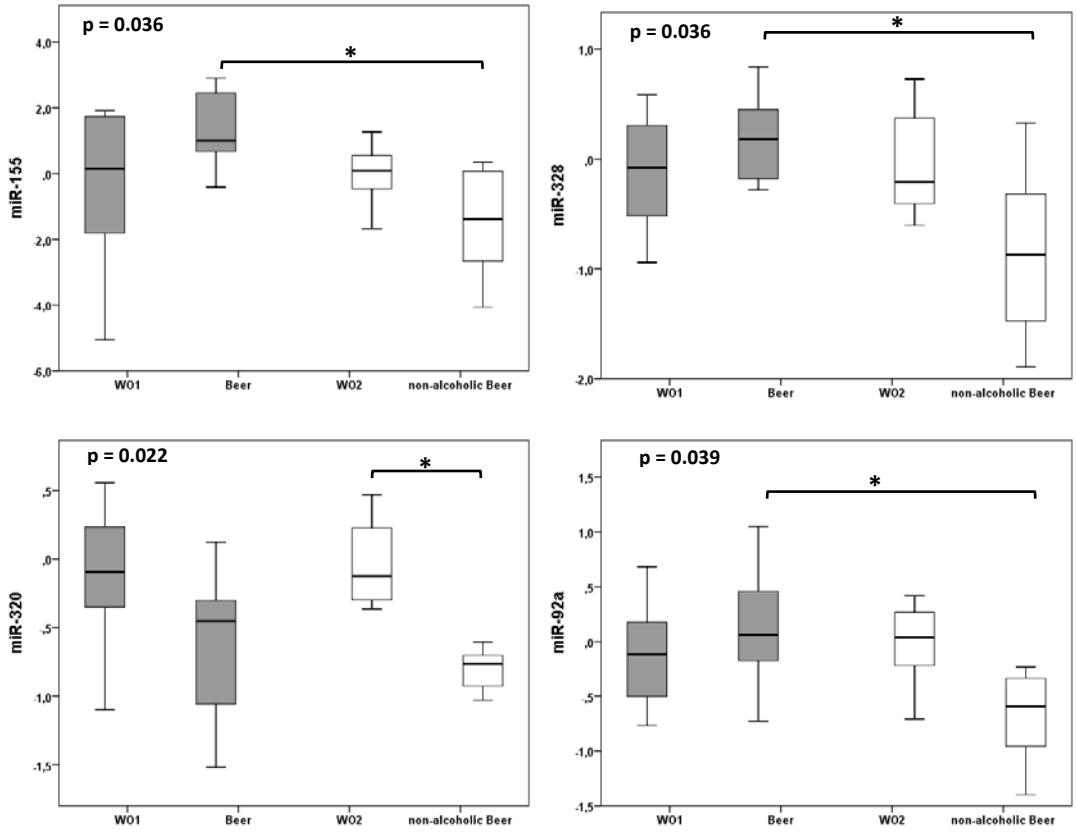

Figure 25. Circulating microRNAs modulated by beer intake. Box plots showing Log_2 transformed relative quantification levels of indicated microRNAs along the intervention. Plasma microRNA levels were calculated with the $2^{-\Delta\Delta Ct}$ method comparing with each WO time-point. P value refers to the intra-subjects comparison of the paired T-student test. * $p<0.05$. WO1: washout 1, Beer: alcoholic beer intervention, WO2: washout 2, non-alcoholic beer: non-alcoholic beer intervention. Data represent mean ± SEM

Additionally, we observed a borderline significant modulation of two microRNAs, miR-107 and miR-17 **(figure 26).** miR-17, a microRNA belonging to miR-17-92 cluster was repressed after both interventions (p= 0.076). However, moiR-107 levels increased after every intervention (p= 0.066)

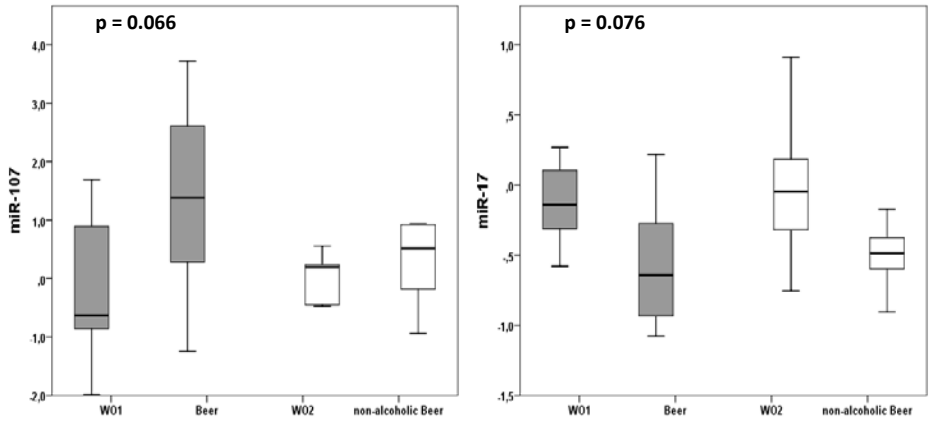

Figure 26. Circulating levels of miR-17 and miR-107 after by beer intake. Box plots showing Log_2 transformed relative quantification levels of indicated microRNAs along the intervention. Plasma microRNA levels were calculated with the $2^{-\Delta\Delta Ct}$ method comparing with each WO time-point. P value refers to the intra-subjects comparison of the paired T-student test. * p<0.05. WO1: washout 1, Beer: alcoholic beer intervention, WO2: washout 2, non-alcoholic beer: non-alcoholic beer intervention. Data represent mean ± SEM

3.4. Post-intervention modification of macrophages microRNA levels with alcoholic and non-alcoholic beer intake.

Several microRNAs present interesting responses to alcoholic and non-alcoholic beer intake with an opposite response to every type of beer in macrophages **(figure 27a).** MiR-145 is highly overexpressed after alcoholic beer ingestion while it does not change after non-alcoholic beer intake (p=0.038) **(figure 27b)**. On the other hand, we observed that the expression of miR-17 (p=0.05), miR-20a (p= 0.03), miR-223 (p=0.011) and miR-26b (p=0.05) increased after alcoholic beer intake while decreased after non-alcoholic beer intake **(figure 27b).**

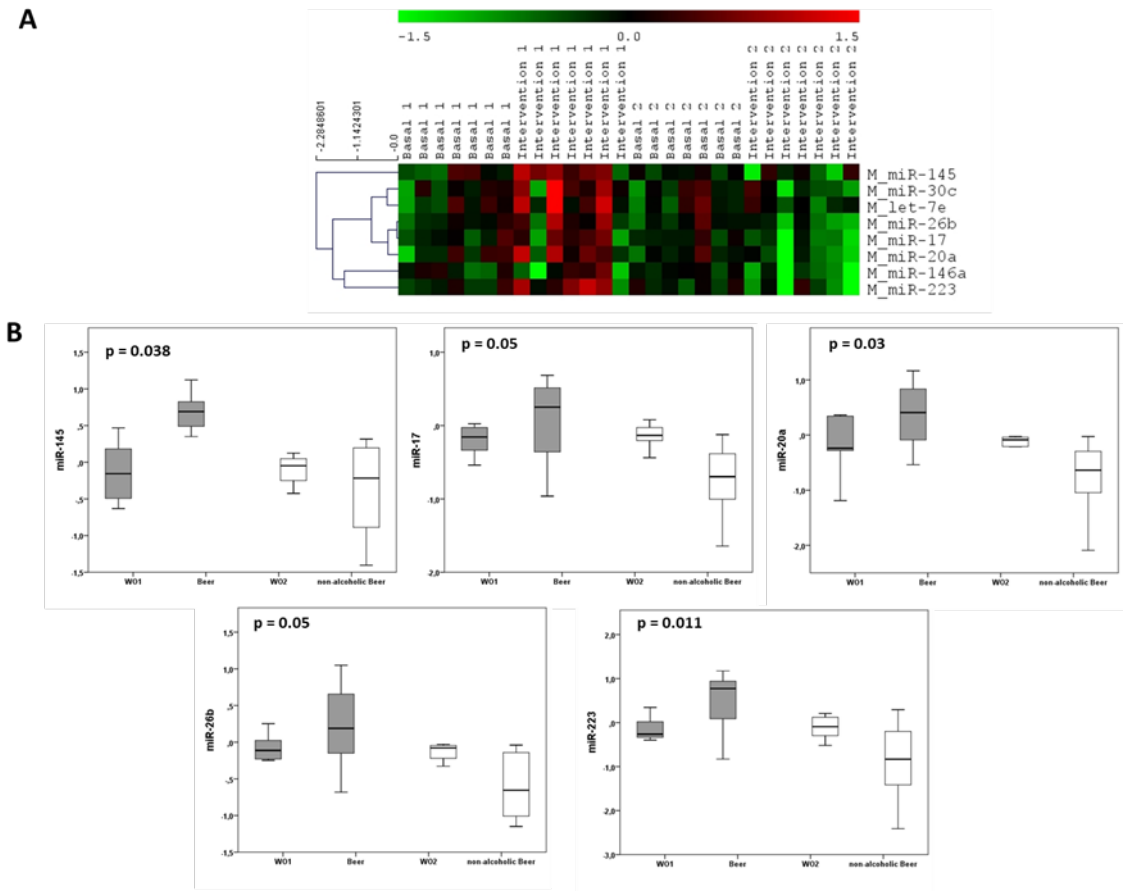

Figure 27. microRNAs modulated by beer intake in macrophages. a) Heat map showing the clustered expression changes after every time-point. b) Box plots showing Log2 transformed relative quantification levels of indicated microRNAs along the intervention. Macrophages microRNA levels were calculated with the 2-ΔΔCt method comparing with each WO time-point. P value refers to the intra-subjects' comparison of the paired T-student test. * p<0.05. WO1: washout 1, Beer: alcoholic beer intervention, WO2: washout 2, non-alcoholic beer: non-alcoholic beer intervention. Data represent mean ± SEM

In addition to this, we observed a similar trend in 3 microRNAs, although their changes did not reach statistical significance. Let 7-e showed a trend to increase its expression after alcoholic beer intake while it does not present changes after non-alcoholic beer intake. In miR-146a we observed a trend to decrease its expression after alcoholic beer intake while miR-30c showed a non-significant increase after alcoholic beer intake and a non-significant decrease after intake of non-alcoholic beer **(figure 28).**

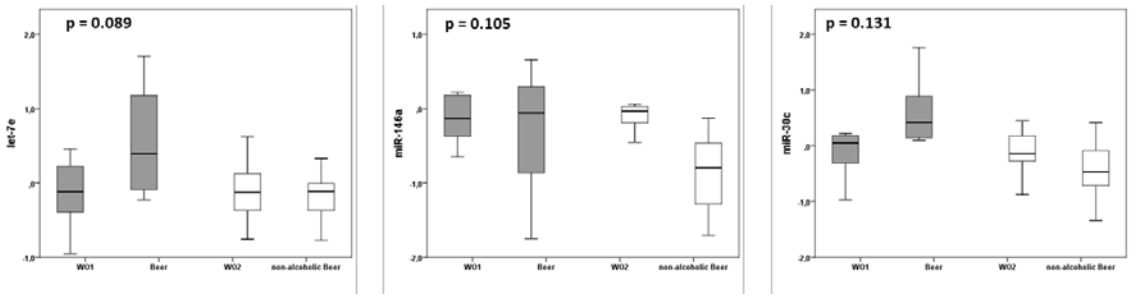

Figure 28. Non-significantly modulated macrophages microRNAs. Box plots showing Log_2 transformed relative quantification levels of indicated microRNAs along the intervention. Plasma microRNA levels were calculated with the $2^{-\Delta\Delta Ct}$ method comparing with each WO time-point. P value refers to the intra-subjects' comparison of the paired T-student test. WO1: washout 1, Beer: alcoholic beer intervention, WO2: washout 2, non-alcoholic beer: non-alcoholic beer intervention. Data represent mean ± SEM

3.5. Correlation between circulating microRNAs levels and macrophages microRNAs levels

We performed different correlation analyses to detect relationships between plasma and macrophage microRNAs modifications after interventions. We obtained interesting positive correlations in miR-328 (p = 0.047 / R^2 = 0.143), let-7e (p = 0.032 / R^2 = 0.165) and miR-26b (p = 0.028 / R^2 = 0.172**) (Figure 29)**.

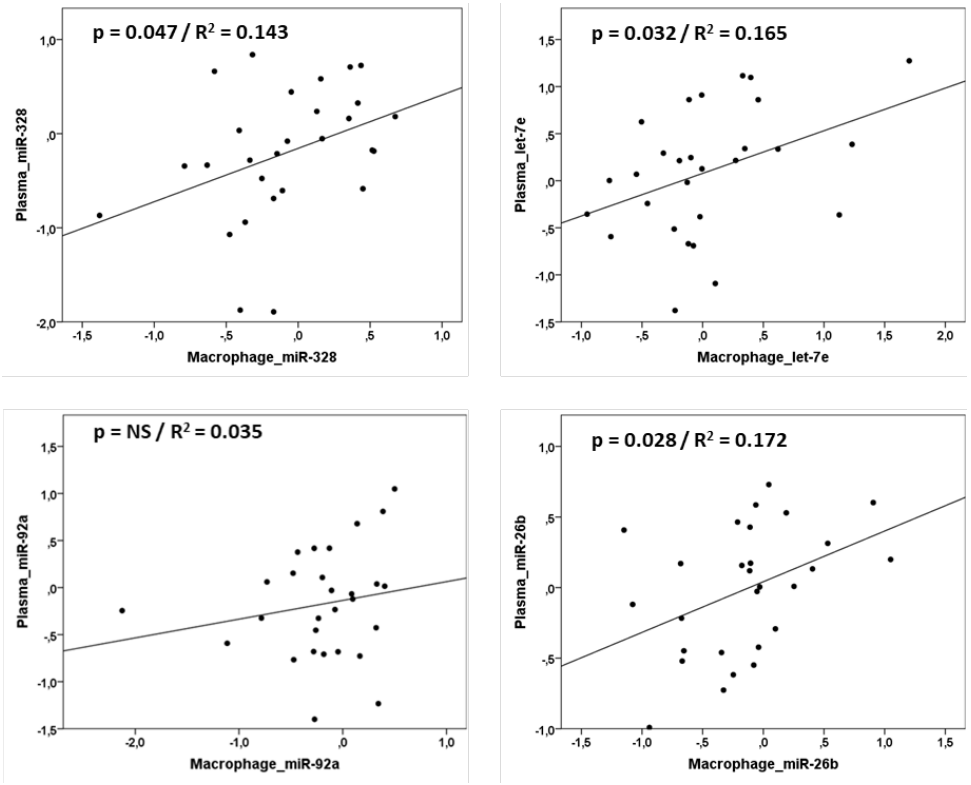

Figure 29. Correlation between microRNAs levels in plasma and macrophages-modulation by beer intake. Plasma and macrophages microRNA levels were calculated with the $2^{-\Delta\Delta Ct}$ method and analysis were performed by Pearson correlation. *** p<0.05**

Other correlated microRNAs were: plasma miR-20a with macrophage miR-26b (p = 0.002 / R^2 = 0.192), plasma miR-26b with macrophage let-7e (p = 0.004 / R^2 = 0.153) and plasma miR-92a with several macrophage microRNAs such as let-7e (p = 0.001 / R^2 = 0.347), miR-26b (p = 0.001 / R^2 = 0.335) and miR-30c (p = 0.004 / R^2 = 0.271). In addition to this, it is important to highlight

the correlation between plasma miR-92a with macrophage miR-17 (p = 0.038 / R^2 = 0.155) and macrophage miR-20a (p = 0.0005 / R^2 = 0.263) given that these 3 microRNAs belong to the same cluster **(figure 30).**

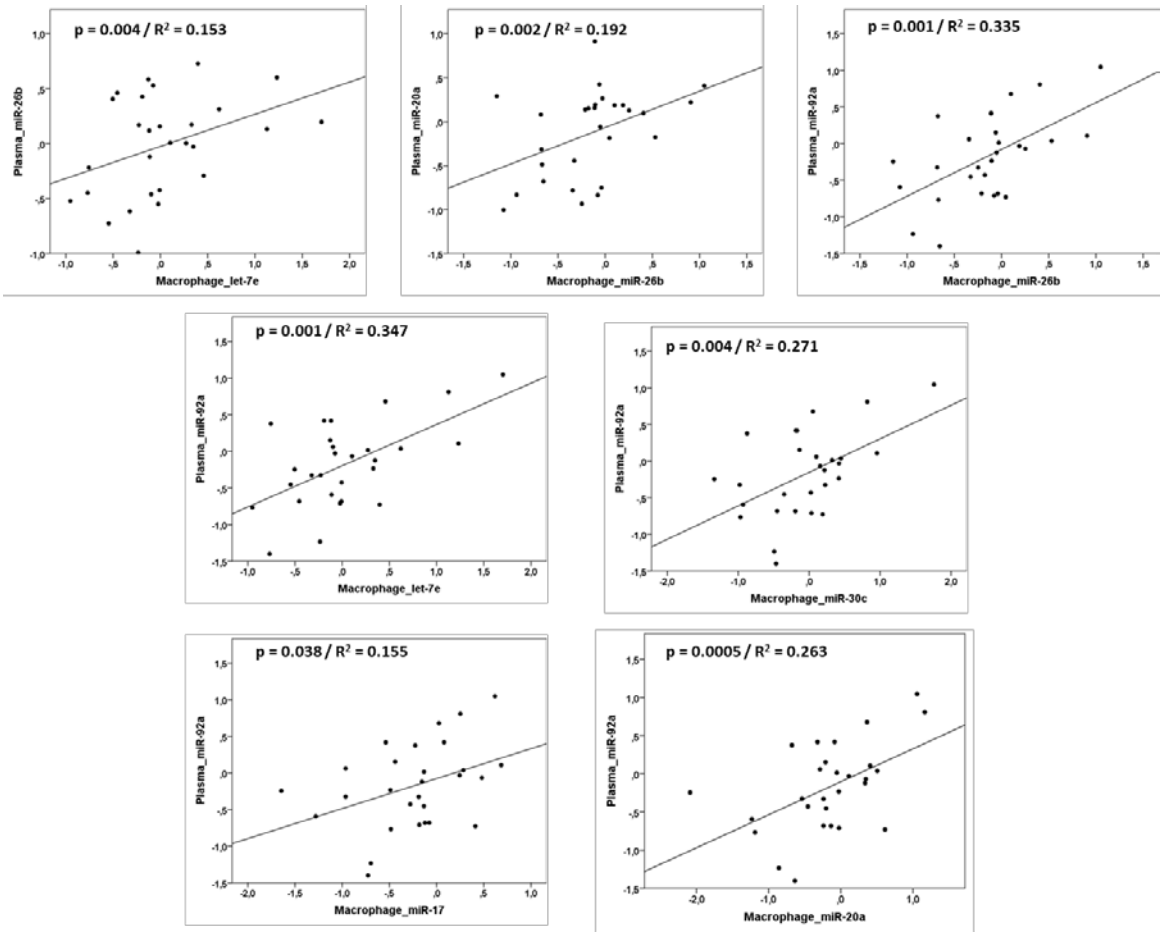

Figure 30: Correlation between microRNAs levels in plasma and macrophages. Plasma and macrophages microRNA levels were calculated with the $2^{-\Delta\Delta Ct}$ method and analysis were performed by Pearson correlation between macrophages and plasma microRNAs differentially expressed after treatment. *** p<0.05**

3.6. Correlation between circulating microRNAs levels and biochemistry parameters

Finally, we performed correlation analyses between microRNA levels significantly modulated by beer and non-alcoholic beer intake and biochemistry and diet parameters. We only obtained one positive correlation between circulating miR-320 levels and blood LDL cholesterol levels (p= 0.028, R^2= 0.202) **(figure 31).**

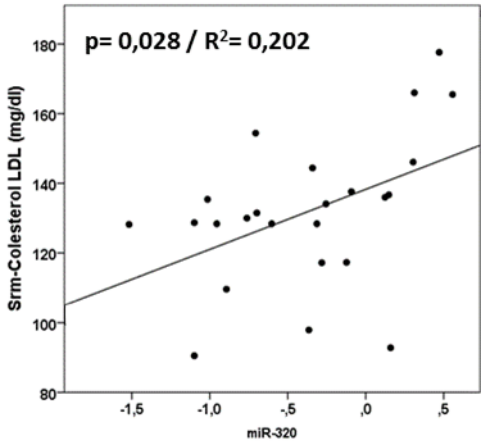

Figure 31: Correlation between circulating mIR-320 and blood LDL cholesterol levels. Analysis were performed by Pearson correlation between blood biochemistry parameters and microRNAs levels. * p<0.05

3.7. Correlation between macrophages microRNAs levels and diet parameters

Different correlations between macrophage let-7 levels and diet parameter were observed. More specifically, macrophage let-7a levels were negatively correlated with soluble fiber intake ($p = 0.027$ / $R^2 = 0.091$), B1 vitamin ($p = 0.029$ / $R^2 = 0.103$) and iron ($p = 0.039$ / $R^2 = 0.048$) **(figure 32).**

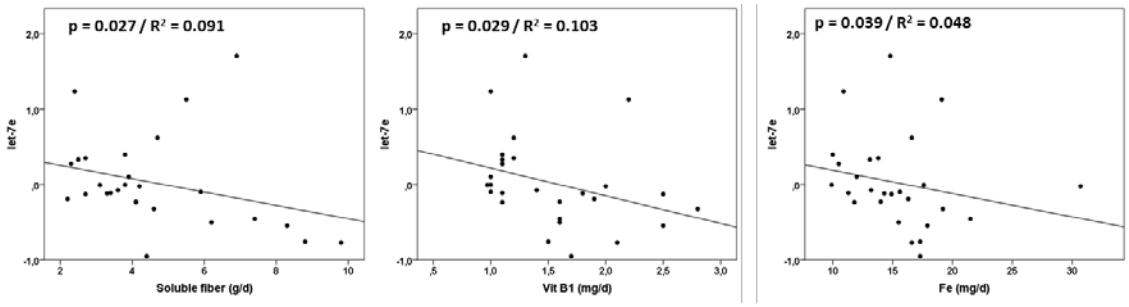

Figure 32: Correlation between macrophage let-7e levels and diet parameters. microRNA levels were calculated with the $2^{-\Delta\Delta Ct}$ method while diet parameters were measure by 3-day die questionnaire. Soluble fiber is expressed in grams per day and B1 vitamin and iron are expressed in milligrams per day. Analysis were performed by Pearson correlation between microRNA levels and micronutrients measured with 3-days diet questionnaire. *** p<0.05**. Vit B1: B1 vitamin, Fe: Iron

3.8. In silico functional analyses

We performed a functional analysis of the microRNAs differentially expressed in order to define the pathways and biological processes that they regulate after beer intake. For that purpose, we used miRWalk database to select the genes that are predicted and validated target of at least 2/4 microRNAs in plasma and 2/5 microRNAs in macrophages.

Functional analyses showed that 585 genes were targets of microRNAs modulated by beer intake in plasma **(figure 33a)**. Targeted genes were mainly related to different types of cancer and related processes (p-53 signalling and cell cycle progression) or nutrient sensing pathways

as PI3K-AKT signaling pathway or AMPK signaling pathway **(figure 33b).** Interestingly**,** *IGF1R*, a gene involved in insulin signalling cascade and *EEF2* (Eukaryotic Translation Elongation Factor 2) which is implicated in protein kinase binding were targets of 3 significantly modulated microRNAs (miR-155, miR-320 and miR-92) **(table 13).**

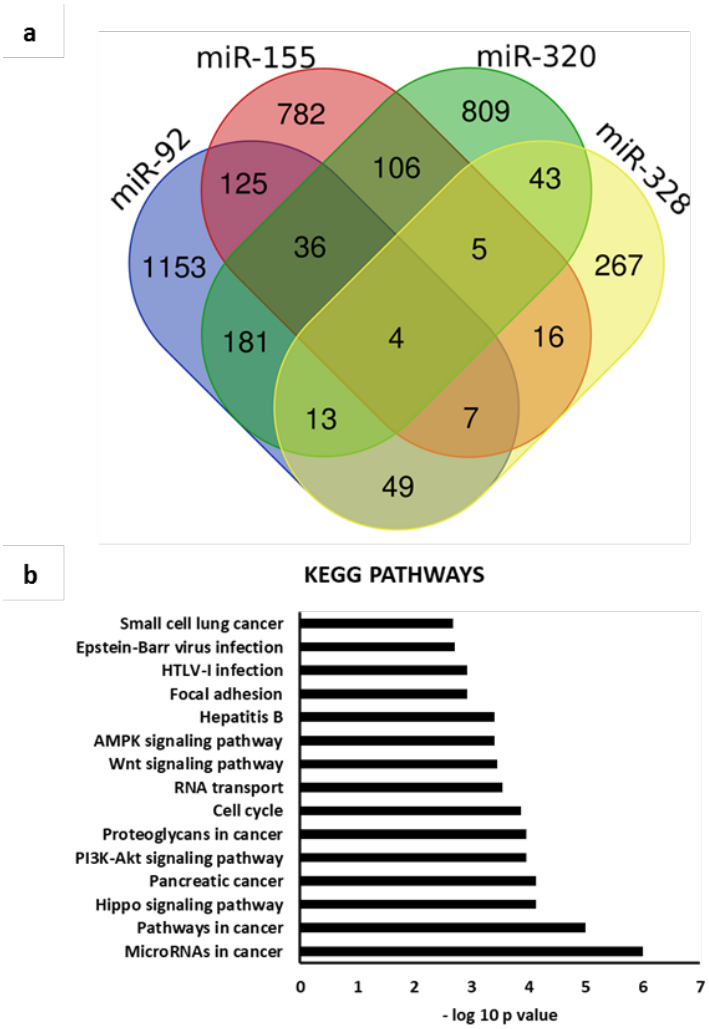

Figure 33. Functional analyses of plasma microRNAs significantly modulated by alcoholic-beer: a) Venn Diagram showing common targets of the modulated microRNAs and b) Bar chart of the top 15 most represented KEGG pathways ($-\log_{10}$ p value adjusted by FDR)

Table 13: Genes commonly targeted by at least 3 microRNAs in plasma after beer intake

Gene symbol	Gene name	GO Biological Process
miR-155 + miR-320 + miR-328 + miR-92		
MYO1D	Myosin ID	actin binding and calmodulin binding
NUFIP2	Nuclear Fragile X Mental Retardation Protein Interacting Protein 2	RNA binding
IGF1R	Insulin Like Growth Factor 1 Recepto	identical protein binding and protein kinase activity
SOX11	SRY-Related HMG-Box Gene 1	DNA binding transcription factor activity and RNA polymerase II core promoter sequence-specific DNA binding
miR-155 + miR-320 + miR-92		
PAPOLA	Polynucleotide Adenylyltransferase Alpha	RNA binding and manganese ion binding
SLC12A4	Solute Carrier Family 12 Member 4	protein kinase binding and cation:chloride symporter activity
LGI2	Leucine Rich Repeat LGI Family Member 2	extracellular region
RAD23B	RAD23 Homolog B, Nucleotide Excision Repair Protei	single-stranded DNA binding and polyubiquitin modification-dependent protein binding
LCOR	Ligand Dependent Nuclear Receptor Corepressor	DNA binding transcription factor activityand transcription factor binding
SLC30A7	Solute Carrier Family 30 Member 7	cation transmembrane transporter activity
NFAT5	Nuclear Factor Of Activated T Cells 5	DNA binding transcription factor activity and transcriptional activator activity, RNA polymerase II proximal promoter sequence-specific DNA binding
SLC7A11	Solute Carrier Family 7 Member 11	amino acid transmembrane transporter activity and cystine:glutamate antiporter activity
MYC	MYC Proto-Oncogene, BHLH Transcription Factor	DNA binding transcription factor activity and RNA polymerase II proximal promoter sequence-specific DNA binding.
CCSER2	Coiled-Coil Serine Rich Protein 2	microtubule bindin
NUCKS1	Nuclear Casein Kinase And Cyclin Dependent Kinase Substrate 1	transcriptional activator activity, RNA polymerase II proximal promoter sequence-specific DNA binding, chromatin binding
ANKH	ANKH Inorganic Pyrophosphate Transport Regulator	inorganic phosphate transmembrane transporter activity and inorganic diphosphate transmembrane transporter activity
RRP15	Ribosomal RNA Processing 15 Homolog	preribosome, large subunit precursor
BRWD1	Bromodomain And WD Repeat Domain Containing 1	molecular function and protein binding
CTNNBL1	Catenin Beta Like 1	binding and enzyme binding
VCPIP1	Valosin Containing Protein Interacting Protein 1	thiol-dependent ubiquitin-specific protease activity
EXOC5	Exocyst Complex Component 5	protein N-terminus binding and Ral GTPase binding
MYO6	Myosin VI	actin binding and actin filament binding
TWF1	Twinfilin Actin Binding Protein 1	protein tyrosine kinase activity and phosphatidylinositol-4,5-bisphosphate binding
LARS	Leucyl-TRNA Synthetase	nucleotide binding and aminoacyl-tRNA editing activity.
RAB14	Member RAS Oncogene Family 14	GTP binding and GDP binding
MORC3	MORC Family CW-Type Zinc Finger 3	protein binding and zinc and metal ion binding
AKAP10	A-Kinase Anchoring Protein 10	protein binding, protein kinase A binding and kinase activity
SLC1A2	Solute Carrier Family 1 Member 2	L-glutamate transmembrane transporter activity and glutamate:sodium symporter activity
SMAD4	SMAD Family Member 4	DNA binding transcription factor activity and sequence-specific DNA binding
RP2	ARL3 GTPase Activating Protein	GTP binding and GTPase activator activity
XPR1	Xenotropic And Polytropic Retrovirus Receptor 1	G-protein coupled receptor activity and transmembrane signaling receptor activity
CREB3L2	CAMP Responsive Element Binding Protein 3 Like 2	DNA binding transcription factor activity and RNA polymerase II proximal promoter sequence-specific DNA binding
EEF2	Eukaryotic Translation Elongation Factor 2	protein kinase binding
XPO1	Exportin 1	RNA binding and transporter activity
DCP2	Decapping MRNA 2	RNA binding and manganese ion binding
EIF3C	Eukaryotic Translation Initiation Factor 3 Subunit C	translation initiation factor activity
NFATC2IP	Nuclear Factor Of Activated T Cells 2 Interacting Protein	protein binding and protein tag
CEP41	Centrosomal Protein 41	microtubule organizing center,centrosome
NAA25	N(Alpha)-Acetyltransferase 25, NatB Auxiliary Subunit	peptide alpha-N-acetyltransferase activity and protein binding
BCAT1	Branched Chain Amino Acid Transaminase 1	identical protein binding and L-leucine transaminase activity
miR-155 + miR-328 + miR-92		
SERTAD2	SERTA Domain Containing 2	transcription coactivator activity
NKX3-1	NK3 Homeobox 1	DNA binding transcription factor activity and transcription factor binding.
TRAK2	Trafficking Kinesin Protein 2	signaling receptor binding and GABA receptor binding
SLC7A1	Solute Carrier Family 7 Member 1	amino acid transmembrane transporter activity and L-ornithine transmembrane transporter activity
PTPRJ	Protein Tyrosine Phosphatase, Receptor Type J	protein kinase binding and protein tyrosine phosphatase activity
SCD	Stearoyl-CoA Desaturase	iron ion binding and stearoyl-CoA 9-desaturase activity
NFIX	Nuclear Factor I X	DNA binding transcription factor activity and transcription factor activity, RNA polymerase II distal enhancer sequence-specific binding
miR-320 + miR-328 + miR-92		
PIKFYVE	Phosphoinositide Kinase, FYVE-Type Zinc Finger Containing	phosphatidylinositol phosphate kinase activity and phosphatidylinositol-3,5-bisphosphate 5-phosphatase activity
YWHAQ	Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Theta	protein domain specific binding and protein N-terminus binding
NEO1	Neogenin 1	cadherin binding
ATXN1	Ataxin 1	identical protein binding and chromatin binding
SMC1A	Structural Maintenance Of Chromosomes 1A	chromatin binding
SLC38A2	Solute Carrier Family 38 Member 2	symporter activity and amino acid transmembrane transporter activity
SPEN	Spen Family Transcriptional Repressor	nucleic acid binding and DNA binding transcription factor activity
TECPR2	Tectonin Beta-Propeller Repeat Containing 2	autophagy
TXLNA	Taxilin Alpha	cytokine activity and high molecular weight B cell growth factor receptor binding
EPG5	Ectopic P-Granules Autophagy Protein 5 Homolog	cytoplasm
DIAPH1	Diaphanous Related Formin 1	nucleotide binding
DNAJB12	DnaJ Heat Shock Protein Family (Hsp40) Member B12	endoplasmic reticulum, membrane and nucleus
miR-155 + miR-320 + miR-328		
PKM	Pyruvate Kinase M1/2	MHC class II protein complex binding
KIAA2018	Upstream Transcription Factor Family Member 3	RNA polymerase II transcription factor activity, sequence-specific DNA binding and protein binding
TMEM33	Transmembrane Protein 33	structural constituent of nuclear pore
FAM199X	Family With Sequence Similarity 199, X-Linked	N.A
TMOD3	Tropomodulin 3	actin binding and tropomyosin binding
DYNC1I1	Dynein Cytoplasmic 1 Intermediate Chain 1	microtubule binding and motor activity

On the other hand, 1729 genes were targets of microRNAs significantly modulated by beer intake in macrophages **(figure 34a)**. Targeted genes were mainly related to cancer but, again, with nutrient sensing pathways as PI3K-AKT signaling pathway or AMPK signaling pathway **(figure 34b)**. *PKFB2* (6-phosfofructo-2-kinase / fructose-2,6-biphosphatase 2), with a role in nutrient sensing is target of 4 modulated macrophage microRNAs. Interestingly, we also observed that genes implicated in immune system as *CD28* (cluster of differentiation 28) and *LIF* (Interleukin 6 Family cytokine) and genes implicated in cholesterol metabolism like *ABCA1* or *ACSL4* (Acyl-CoA Synthetase Long Chain Family Member 4) are targets of these microRNAs **(table 14).**

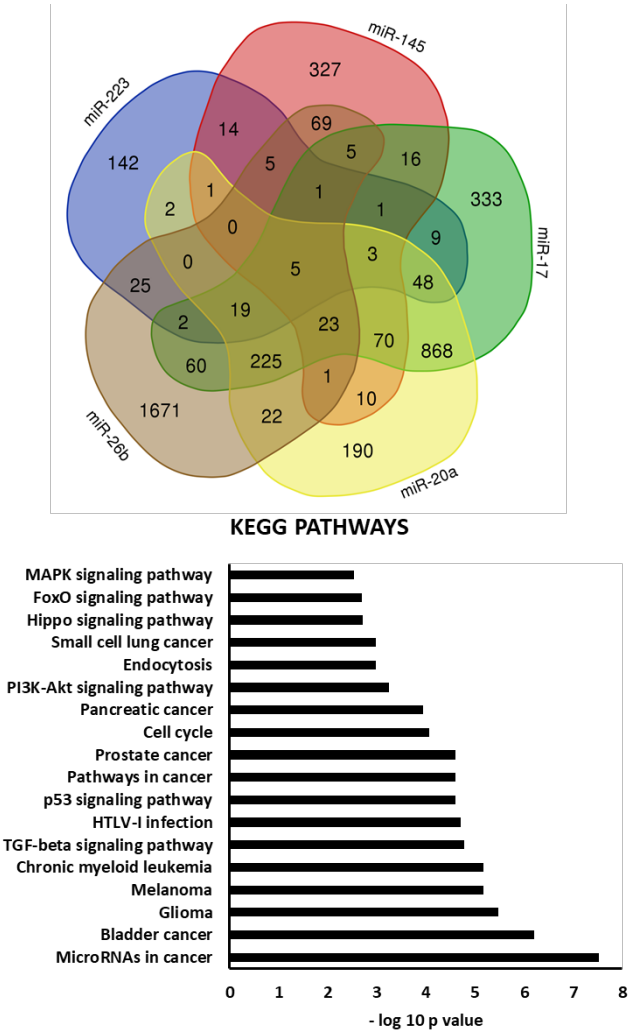

Figure 34. Functional analyses of significantly modulated macrophage microRNAs. a) Venn Diagram showing common targets of the modulated microRNAs and b) Bar chart of the top 15 most represented KEGG pathways (-$\log_{10}$ p value adjusted by FDR).

Table 14: Genes commonly targeted by at least 3 microRNAs in macrophages after beer intake

Gene symbol	Gene name	GO Biological Process
miR-145 + miR-17 + miR-20a + miR-223 + miR-26b		
MDM2	MDM2 Proto-Oncogene	identical protein binding and ligase activity
DENND5B	DENN Domain Containing 5B	calcium channel activity and Rab guanyl-nucleotide exchange factor activity.
ANKRD52	Ankyrin Repeat Domain 52	N.A
PURB	Purine Rich Element Binding Protein B	RNA binding
ST8SIA3	ST8 Alpha-N-Acetyl-Neuraminide Alpha-2,8-Sialyltransferase 3	sialyltransferase activity and alpha-N-acetylneuraminate alpha-2,8-sialyltransferase activity
miR-145 + miR-17 + miR-20a + miR-223		
TMOD2	Tropomodulin 2	actin binding and tropomyosin binding
SLC1A2	Solute Carrier Family 1 Member 2	L-glutamate transmembrane transporter activity and glutamate:sodium symporter activity
SCN3A	Sodium Voltage-Gated Channel Alpha Subunit 3	ion channel activity and voltage-gated sodium channel activity.
miR-145 + miR-17 + miR-223 + miR-26b		
SCN2A	Sodium Voltage-Gated Channel Alpha Subunit 2	ion channel activity and voltage-gated sodium channel activity
miR-17 + miR-20a + miR-223 + miR-26b		
POLR3G	RNA Polymerase III Subunit G	DNA-directed 5-3 RNA polymerase activity and RNA polymerase III activity
FAM199X	Family With Sequence Similarity 199, X-Linked	N.A
PFKFB2	6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 2	protein kinase binding and 6-phosphofructo-2-kinase activity
MSMO1	Methylsterol Monooxygenase 1	oxidoreductase activity and C-4 methylsterol oxidase activity
TMEM64	Transmembrane Protein 64	endoplasmic reticulum and integral component of membrane
HECA	Hdc Homolog, Cell Cycle Regulator	nucleus, cytoplasm and membrane
RRAS2	RAS Related 2	GTP binding and GTPase activity
SV2B	Synaptic Vesicle Glycoprotein 2B	transporter activity and transmembrane transporter activity
NIPA1	NIPA Magnesium Transporter 1	magnesium ion transmembrane transporter activity
F3	Coagulation Factor III	phospholipid binding and cytokine receptor activity
LIF	Interleukin 6 Family Cytokine	signaling receptor binding and growth factor activity
ZBTB18	Zinc Finger And BTB Domain Containing 18	DNA binding transcription factor activity and sequence-specific DNA binding
BBX	HMG-Box Containing	RNA polymerase II transcription factor activity, sequence-specific DNA binding
C9orf40	Chromosome 9 Open Reading Frame 4	RNA polymerase II transcription factor activity, sequence-specific DNA binding
EPM2AIP1	EPM2A Interacting Protein 1	RNA polymerase II transcription factor activity, sequence-specific DNA binding and protein binding
MEF2D	Myocyte Enhancer Factor 2D	DNA binding transcription factor activity and RNA polymerase II transcription factor activity, sequence-specific DNA binding
ZNF148	Zinc Finger Protein 148	nucleic acid binding and sequence-specific DNA binding
CMPK1	Cytidine/Uridine Monophosphate Kinase	kinase activity and nucleobase-containing compound kinase activity
CREBRF	CREB3 Regulatory Factor	DNA binding transcription factor activity and sequence-specific DNA binding
miR-145 + miR-17 + miR-20a + miR-26b		
BTG2	BTG Anti-Proliferation Factor 2	transcriptional activator activity, RNA polymerase II proximal promoter sequence-specific DNA binding
NTN4	Netrin 4	laminin-1 binding
CD28	CD28 Molecule	identical protein binding and SH3/SH2 adaptor activity
FNDC3B	Fibronectin Type III Domain Containing 3B	RNA and protein binding
NUFIP2	FMR1 Interacting Protein	RNA binding
SLC25A44	Solute Carrier Family 25 Member 44	transmembrane transporter activity
SAMD8	Sterile Alpha Motif Domain Containing 8	transferase activity
NAA50	N(Alpha)-Acetyltransferase 50, NatE Catalytic Subunit	N-acetyltransferase activity and H4 histone acetyltransferase activity
SMAD4	SMAD Family Member 4	DNA binding transcription factor activity and sequence-specific DNA binding
PLAG1	PLAG1 Zinc Finger	DNA binding transcription factor activity and transcriptional activator activity, RNA polymerase II proximal promoter sequence-specific DNA binding
RAB11A	Member RAS Oncogene Family	GTP binding and microtubule binding
CNOT6L	CCR4-NOT Transcription Complex Subunit 6 Like	poly(A)-specific ribonuclease activity
RNF216	Ring Finger Protein 216	ligase activity
ZNF426	Zinc Finger Protein 426	nucleic acid binding
RNF24	Ring Finger Protein 24	ubiquitin protein ligase activity, protein and metal ion binding
ZBTB25	Zinc Finger And BTB Domain Containing 25	DNA binding transcription factor activit
FBXO28	F-Box Protein 28	protein binding
ACSL4	Acyl-CoA Synthetase Long Chain Family Member 4	long-chain fatty acid-CoA ligase activity and arachidonate-CoA ligase activity
SAMD12	Sterile Alpha Motif Domain Containing 12	sequence-specific DNA binding
ABCA1	ATP Binding Cassette Subfamily A Member 1	signaling receptor binding and phospholipid binding
ABCG4	ATP Binding Cassette Subfamily G Member 4	protein homodimerization activity and ATPase activity
PBX3	PBX Homeobox 3	DNA binding transcription factor activity and sequence-specific DNA binding
HMGA2	High Mobility Group AT-Hook 2	enzyme binding and transcriptional activator activity, RNA polymerase II proximal promoter sequence-specific DNA binding

3.9. Genes network analysis

We performed interaction network analysis to check how linked are the genes targeted by the regulated microRNAs after beer intake. While the network analysis of genes in plasma showed a big cluster of related genes **(figure 35a**), genes targeted by modulated microRNAs in macrophages showed two different interaction nodes linked each other **(figure 35b).** The core of the node of genes targeted by plasma microRNAs is composed by *CHD9* (Chromodomain helicase DNA binding protein 9), *ZNF148* (Zinc finger protein 148), *MEF2D* (Myocyte enhancer factor 2D), *CSNK1A1* (Casein Kinase 1 Alpha 1), *CNOT6L* (CCR4-NOT transcription complex, subunit 6-like), *HDAC4* (Histone deacetylase 4), *TNRC6A* (Trinucleotide repeat containing 6A),

ABCA1 and *PPARα* (Peroxisome proliferator-activated receptor alpha) , involved in negative regulation of macrophage derived foam cell differentiation and in the negative regulation of glycolytic process and cholesterol storage.

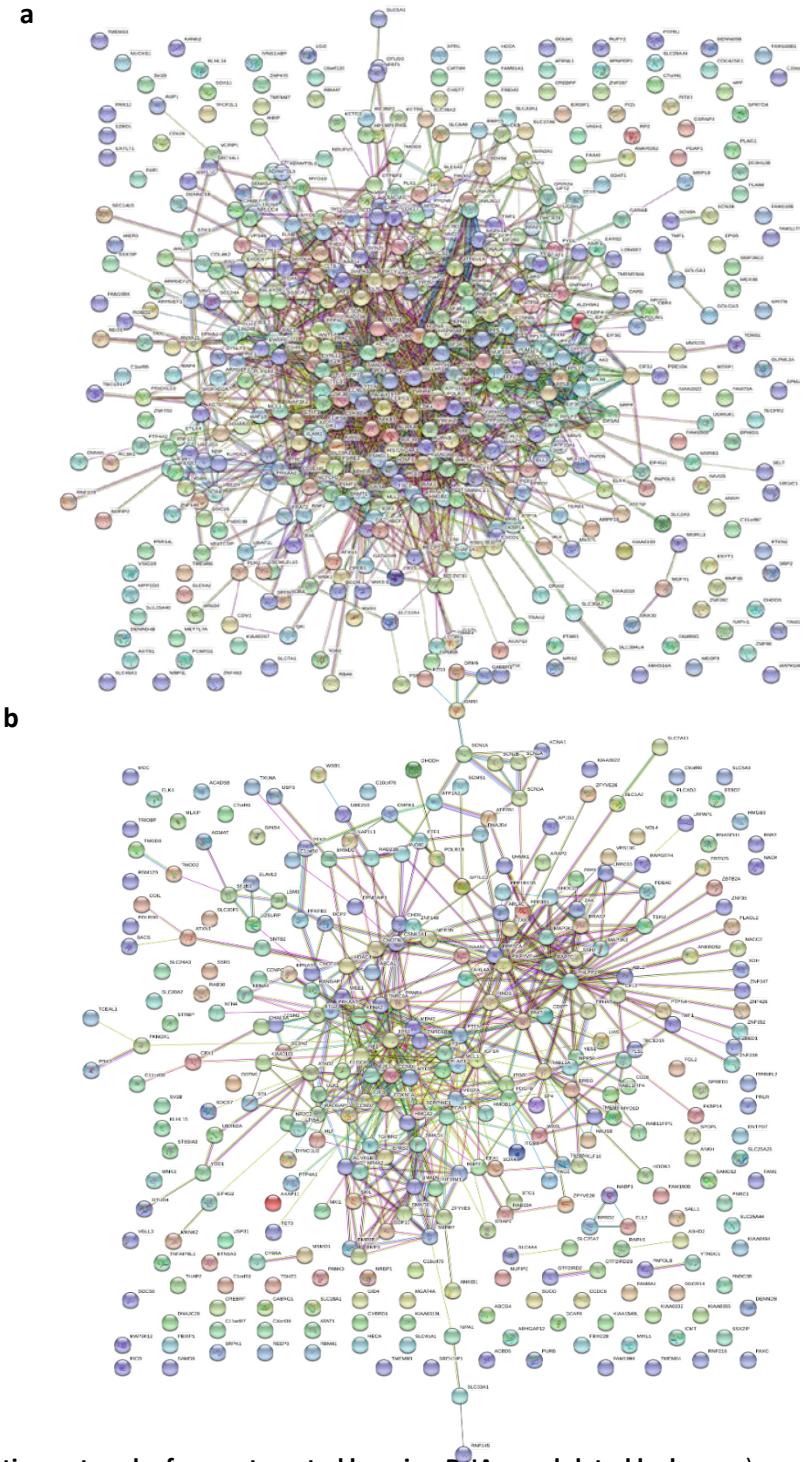

Figure 35 Interaction network of genes targeted by microRNAs modulated by beer. a) genes targeted by significantly modulated plasma microRNAs and b) genes targeted by significantly modulated macrophage microRNAs

An in depth analysis of the two nodes of the macrophage network using KEGG pathways showed that the genes in the right node are related to immune system (T cell receptor signaling pathway

or HTLV-I infection) and axon guidance while genes in the left node are related to cancer, nutrient sensing pathways (PI3K-AKT, FOXO and AMPK signaling) and cell cycle.

4. PREDIMED PLUS STUDY

4.1. Characteristics of the population

We selected 20 patients of each group that were representative of each type of the intervention. The population characteristics are shown in **table 15.**

We observed several changes after 1 year of intervention. We observe a significant decrease in weight ($p < 0.001$), waist ($p < 0.001$), hip ($p < 0.001$) and BMI ($p < 0.001$) in the intervention group. Waist and hip perimeters did not change significantly in the control group, whereas weight ($p < 0.005$) and BMI ($p=0.028$) increased. The body composition did change significantly except for an increase in visceral fat in control group ($p= 0.028$) and a decrease in intervention group ($p= 0.047$). The biochemistry parameters did not show significant changes except for a decrease in triglycerides levels in intervention group ($p= 0.048$). Finally, regarding lifestyles parameters, we observed that both control ($p =0.001$) and intervention ($p =0.002$) increased their adherence to MD. This is not surprising, as the control group is also encouraging to increase adherence to the MD, but without CR. However, regarding PA, which is only introduced in the intervention group, we found a borderline significant increase in percentage of individuals performing moderate and intensive PA at the expense of a decrease in the percentage of individuals in the low PA group ($p = 0.056$).

Table 15: Population description and biochemistry and anthropometric measured during the year of intervention. Values are expressed in mean ± SEM.

		Basal	1 year	p value
General description and health parameters				
Sex (men/women) (%)	Control	65 /35		n.s.
	Intervention	40/ 60		n.s.
Age (years)	Control	62.35 ± 3.55	63.35 ± 3.55	n.s.
	Intervention	65.10 ± 4.71	66.10 ± 4.71	n.s.
Systolic pressure (mmHg)	Control	130.05 ± 17.92	129.65 ± 13.84	n.s.
	Intervention	134.20 ± 13.76	132.20 ± 17.27	n.s.
Diastolic pressure (mmHg)	Control	83.60 ± 12.09	83.65 ± 12.09	n.s.
	Intervention	86.85 ± 16.19	84.95 ± 16.74	n.s.
Anthropometric measures				
Weight (kg)	Control	81.5 ± 12.93	82.45 ± 12.58	0.005
	Intervention	82.58 ± 13.89	79.07 ± 14.32	<0.001
Waist (cm)	Control	101.91 ± 9.49	101.42 ± 9.60	n.s.
	Intervention	103.41 ± 11.82	98.96 ± 12.28	<0.001
Hip (cm)	Control	104.20 ± 4.96	104.10 ± 4.65	n.s.
	Intervention	107.66 ± 9.41	104.30 ± 9.69	<0.001
BMI (kg/m2)	Control	30.10 ± 2.25	30.46 ± 2.30	0.028
	Intervention	31.36 ± 3.50	29.75 ± 3.66	<0.001
Fat mass (%)	Control	34.11 ± 8.64	34.53 ± 8.09	n.s.
	Intervention	37.25 ± 7.95	36.83 ±8.68	n.s.
Muscular mass (%)	Control	28.21 ± 5.16	28.55 ± 4.23	n.s.
	Intervention	27.95 ± 4.83	27.00 ± 4.32	n.s.
Visceral fat	Control	14.45 ± 3.48	15.05 ± 3.53	0.024
	Intervention	14.55 ± 4.20	13.70 ± 3.84	0.047
Biochemistry parameters				
Glucose (mg/dl)	Control	126.00 ± 52.15	136.40 ± 67.41	n.s.
	Intervention	108.65 ± 19.82	102.06 ± 31.38	n.s.
Cholesterol (mg/dl)	Control	191.65 ± 38.26	195.53 ± 44.77	n.s.
	Intervention	188.75 ± 34.16	192.72 ± 34.03	n.s.
HDL cholesterol (mg/dl)	Control	44.74 ± 7.51	45.90 ± 7.80	n.s.
	Intervention	49.05 ± 9.99	51.33 ±10.83	n.s.
LDL cholesterol (mg/dl)	Control	124.33 ± 34.41	119.35 ± 91.912	n.s.
	Intervention	112.45 ± 25.10	116.97 ±26.97	n.s.
Triglycerides (mg/dl)	Control	143.89 ± 81.13	162.40 ± 75.48	n.s.
	Intervention	139.75 ± 44.20	123.67 ± 44.53	0.048
Lifestyle parameters				
17-points MD questionnarie	Control	9.95 ± 1.50	11.83 ± 1.97	0.001
	Intervention	9.75 ± 2.29	12.10 ± 2.36	0.002
PA (%)	Control	Low PA 60% Moderate PA 25% High PA 15 %	Low PA 60% Moderate PA 25% High PA 15 %	n.s.
	Intervention	Low PA 95% Moderate PA 0% High PA 5%	Low PA 75% Moderate PA 15% High PA 10%	0.056
BMI: dody mass index, PA: physical activity, n.s.: non significant				

4.2. Modification of plasma microRNA levels after 1 year of treatment.

Then we analyzed the change in the circulating levels of microRNAs targeting nutrient sensor pathways after 1 year of intervention. We performed a paired significance analysis for

microarray (SAM) test with hierarchical clustering of significant microRNAs applying a FDR using TMEV software. SAM test showed 8 microRNAs modified in control group (miR-200c, miR-93, miR-92a, miR-15b, miR-301, miR-30b, miR-17-5p and miR-130a) and 6 microRNAs in intervention group (miR-195, miR-19a, miR-181a, miR-200c and miR-30b, miR-301). However, the FDR indicated that in control group included 2.5 possible false positives while the intervention group included 2 possible false positives **(figure 36).**

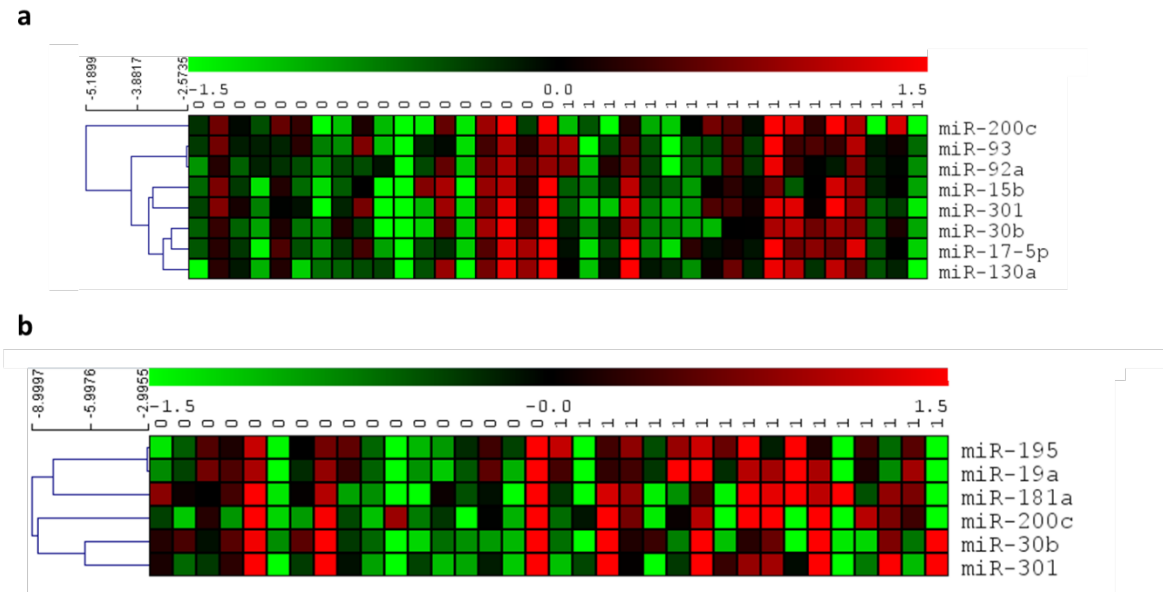

Figure 36: Heat map showing the hierarchical clustering of SAM significant microRNAs whose expression changes in**.** a) Control group hierarchical clustering and b) intervention group hierarchical clustering. Hierarchical clustering was performed with Euclidean distances. Red boxes represent upregulated microRNAs and green boxes represent downregulated microRNAs according to the scale depictured on the top of each panel. 0 means basal time and 1 means 1-year post intervention.

We re-analyzed data using a paired t-test. However, none of those microRNAs were found significantly modulated in any group, although we did observe a trend towards an increase in these microRNAs after 1 year of intervention **(figure 37).**

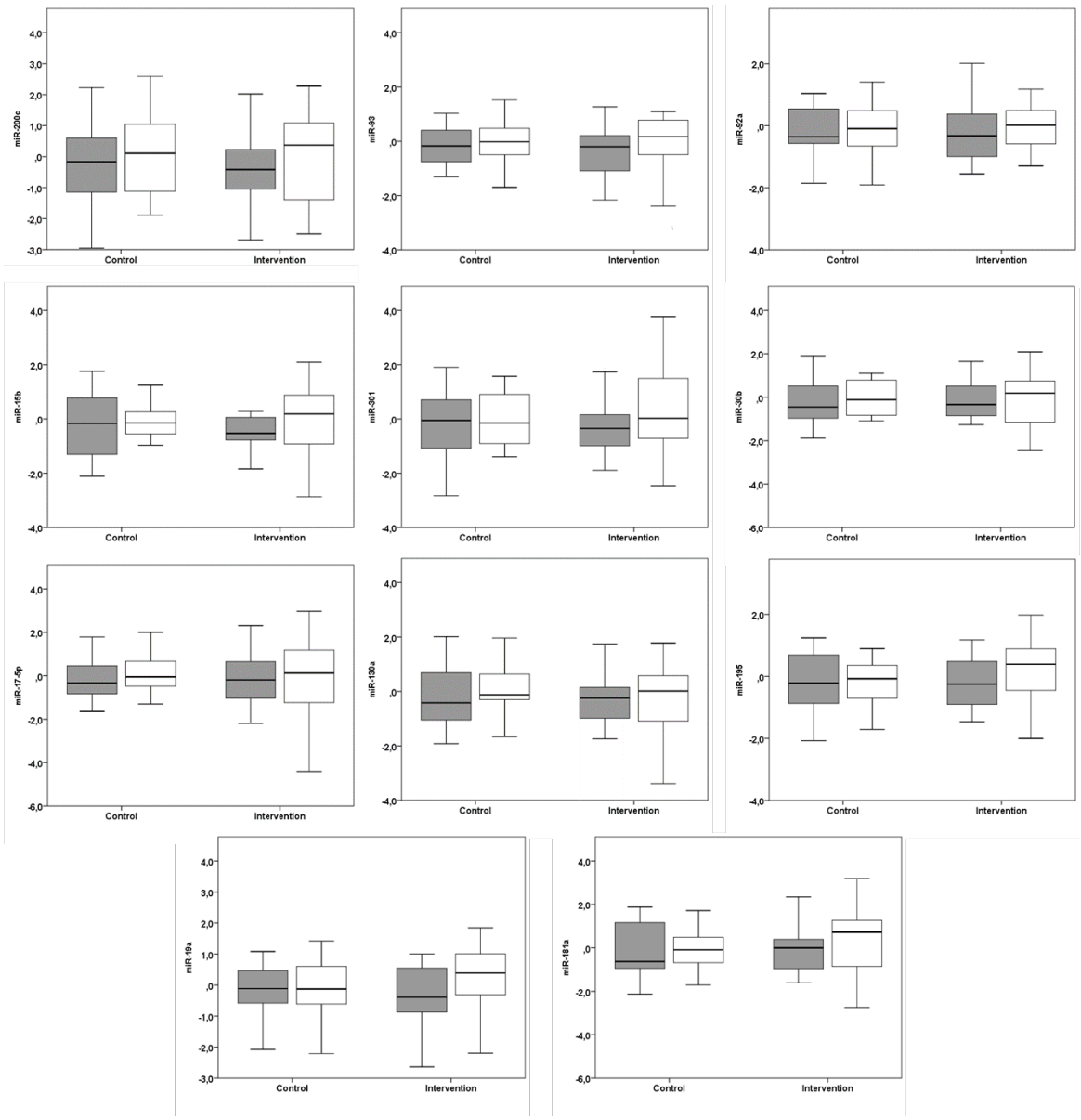

Figure 37. Non-significantly modulated plasma microRNAs. Box plots showing Log_2 transformed relative quantification levels of indicated microRNAs along the 1-year intervention. Plasma microRNA levels were calculated with the $2^{-\Delta\Delta Ct}$ method comparing basal and 1-year time point. MicroRNAs were analysed using an intra-subjects' comparison of the paired T-student test. Data represent mean ± SEM
7

4.3. Modification of macrophages microRNA levels after 1 year of treatment.

Again, we first did a paired SAM analysis with TMEV to compare microRNA levels of basal time and 1-year time in macrophages. We found 6 significant microRNAs in control group with 2 possible false positives (miR-21, miR-195, miR-16, miR-181a, miR-20a and miR-221) **(figure 38a)**. In intervention group, we also observed 6 significant microRNAs (miR-30c, miR-497, miR-130a, miR-152, miR-27b and miR-302c) with 1 possible false positive **(figure 38b).**

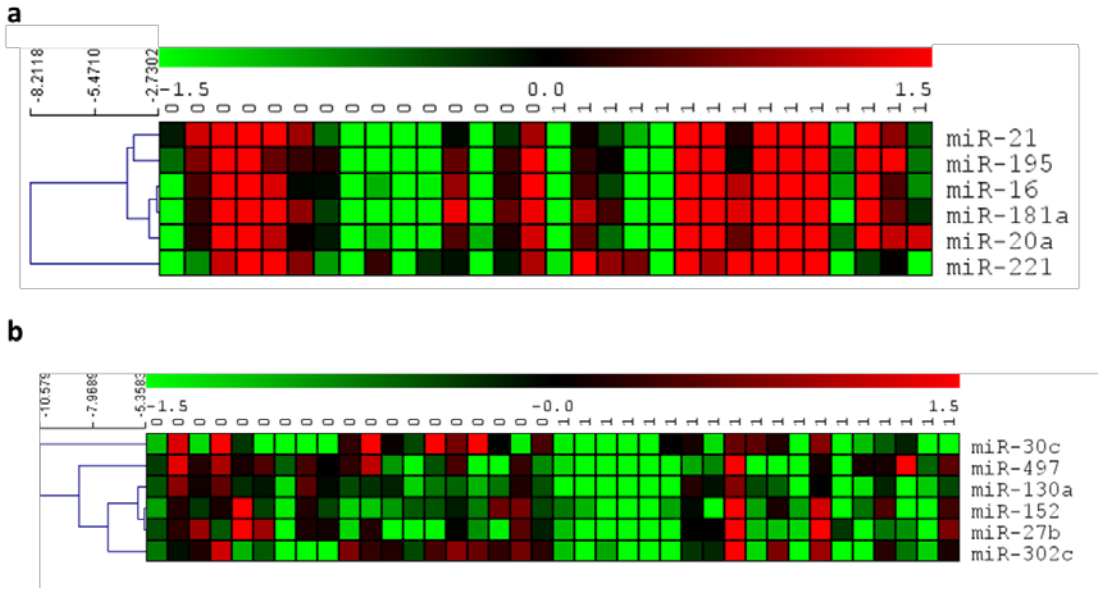

Figure 38: Heat map showing the hierarchical clustering of SAM significant microRNAs whose expression changes in macrophages microRNAs after 1 year of intervention. a) Control group hierarchical clustering presents 2.5 false positives and b) intervention group hierarchical clustering presents 2 false positives. Hierarchical clustering was performed with Euclidean distances. Red boxes represent upregulated microRNAs and green boxes represent downregulated microRNAs according to the scale depictured on the top of each panel. 0 means basal time and 1 means 1-year post intervention.

When we re-analyzed using a paired t-test, none of the significant microRNAs in control group were found to be significantly modulated **(figure 39)**. However, in intervention group, we observed a significant decrease of miR-130a ($p<0.05$) and almost significant decrease in miR-30c ($p=0.07$) while in the other 4 microRNAs the paired t-test did not support the SAM analysis **(figure 39)**

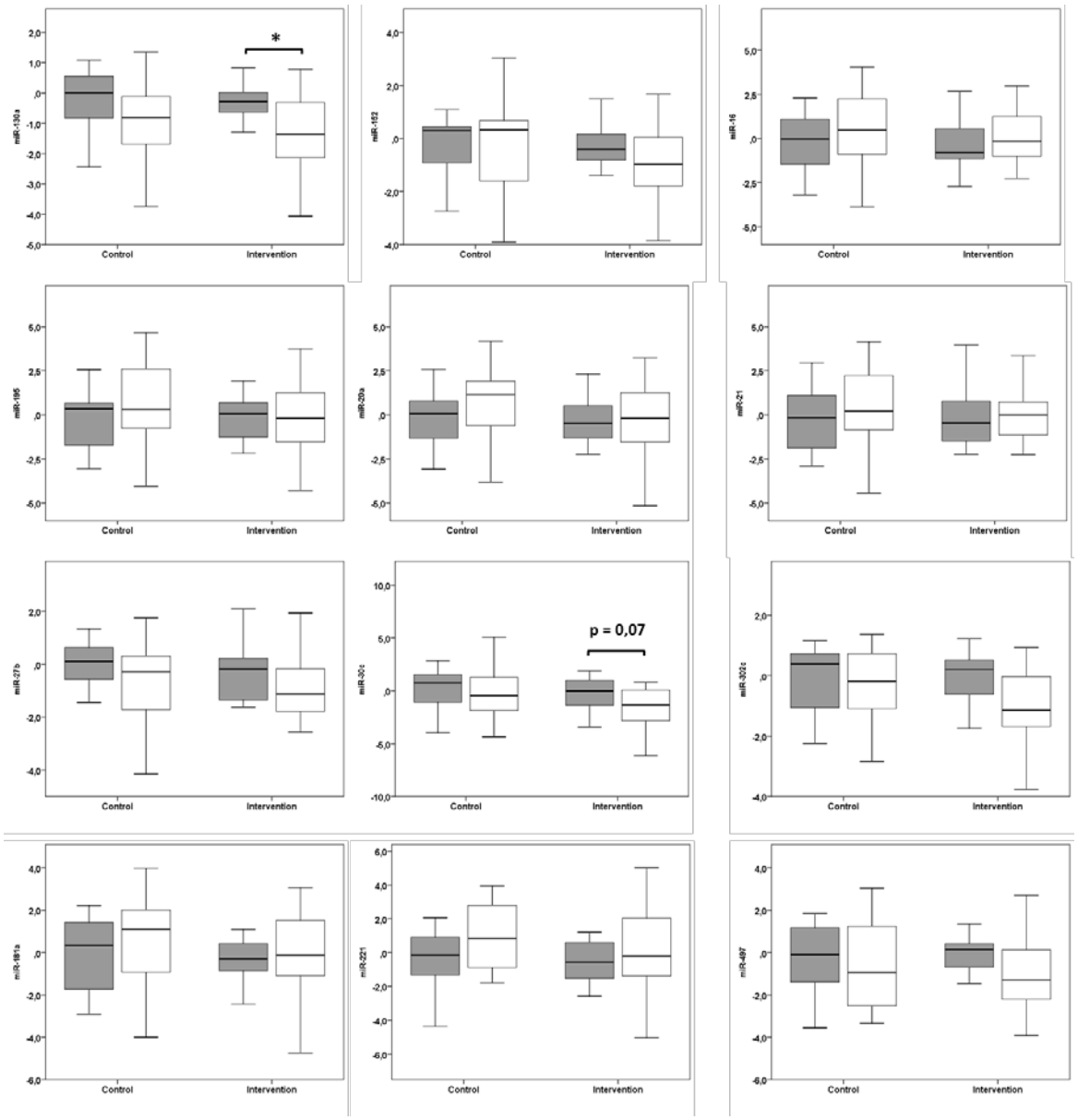

Figure 39. Significant and non-significant modulated macrophages microRNAs. Box plots showing Log_2 transformed relative quantification levels of indicated microRNAs along the 1-year intervention. Macrophages microRNA levels were calculated with the $2^{-\Delta\Delta Ct}$ method comparing basal and 1-year time point. MicroRNAs were analysed using an intra-subjects' comparison of the paired T-student test. ***p<0.05** Data represent mean ± SEM

4.4. In silico functional analysis

We performed a functional analysis of the targets of macrophage miR-130a and miR-30c in order to identify the pathways and biological processes that could be modulated by the intervention Functional analyses showed that 115 genes were common targets of both microRNAs. **(figure 40)**. Pathway enrichment analysis did not show any significant pathway. However, it worth mentioning that some of those targets belong to the nutrient sensor pathways (**table 16).** Specifically A-Kinase Anchoring Protein 10 (*AKAP10),* a gene related to activation of cAMP via PKA (protein kinase A). Interestingly, genes associated with LDL metabolism like LDL receptor

(*LDLR*) and LDL Receptor Related Protein 6 (*LRP6*) are also among the targets of these microRNAs **(table 16).**

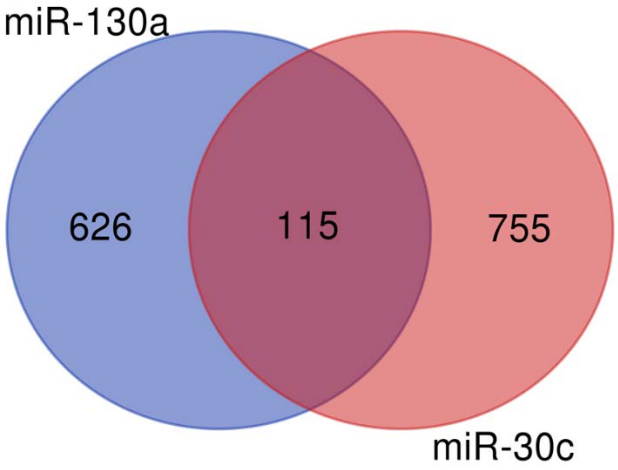

Figure 40: Functional analyses of macrophages microRNAs significantly modulated by the intervention. Venn Diagram showing common targets of the modulated macrophages microRNAs.

Table 16: Genes commonly targeted by miR-130a and miR-30c in macrophages after 1 year of intervention.

Gene symbol	Gene name	GO Biological Process
FAM178A	Family With Sequence Similarity 178, Member A	protein binding, ubiquitin protein ligase binding, protein-containing complex binding
NUFIP2	Nuclear Fragile X Mental Retardation Protein Interacting Protein 2	RNA binding.
TAF4B	TATA-Box Binding Protein Associated Factor 4b	DNA binding transcription factor activity and NF-kappaB binding
ENPP4	Ectonucleotide Pyrophosphatase/Phosphodiesterase 4	sulfuric ester hydrolase activity and bis(5-adenosyl)-triphosphatase activity
DPYSL2	Dihydropyrimidinase Like 2	protein kinase binding and microtubule binding
SOX4	SRY (Sex Determining Region Y)-Box 4	DNA binding transcription factor activity and transcriptional activator activity, RNA polymerase II proximal promoter sequence-specific DNA binding.
NRP1	Neuropilin 1	heparin binding and growth factor binding
LYRM2	LYR Motif Containing 2	protein binding
GJA1	Gap Junction Protein Alpha	ignaling receptor binding and protein domain specific binding
ATG2B	Autophagy Related 2B	autophagy
MAP3K5	Mitogen-Activated Protein Kinase Kinase Kinase 5	protein homodimerization activity and protein kinase activity
PPP1R15B	Protein Phosphatase 1 Regulatory Subunit 15B	protein serine/threonine phosphatase activity
ARHGEF12	Rho Guanine Nucleotide Exchange Factor 12	GTPase activator activity and G-protein coupled receptor binding
ZNF529	Zinc Finger Protein 529	nucleic acid binding
LCOR	Ligand Dependent Nuclear Receptor Corepressor	DNA binding transcription factor activityand transcription factor binding
OPHN1	Oligophrenin 1	actin binding and phospholipid binding
CSF1	Colony stimulating factor 1	protein homodimerization activity and growth factor activity
SLC6A6	Solute Carrier Family 6 Member 6	neurotransmitter:sodium symporter activity and taurine transmembrane transporter activity
ARF4	ADP Ribosylation Factor 4	GTP binding and obsolete signal transducer activity
KBTBD6	Kelch Repeat And BTB Domain Containing 6	protein binding
SCML2	Scm Polycomb Group Protein Like 2	DNA binding transcription factor activity and sequence-specific DNA binding
RAB14	Member RAS Oncogene Family	GTP binding and GDP binding
RPS27A	Ribosomal Protein S27a	structural constituent of ribosome
NACC2	NACC Family Member 2	protein homodimerization activity and transcriptional repressor activity, RNA polymerase II proximal promoter sequence-specific DNA binding
QKI	KH Domain Containing RNA Binding	nucleic acid binding and RNA binding
ARID5B	AT-Rich Interaction Domain 5B	transcription coactivator activityand RNA polymerase II regulatory region sequence-specific DNA binding
HABP4	Hyaluronan Binding Protein 4	RNA and protein binding
HLF	PAR BZIP Transcription Factor	DNA binding transcription factor activity and double-stranded DNA binding
LONRF2	LON Peptidase N-Terminal Domain And Ring Finger 2	ATP-dependent peptidase activity
ZNF711	Zinc Finger Protein 711	sequence-specific DNA binding
ATP6V1C1	ATPase H+ Transporting V1 Subunit C1	transporter activity and proton-transporting ATPase activity, rotational mechanism
C7orf60	Base Methyltransferase Of 25S RRNA 2 Homolog	methyltransferase activity
PFN2	Profilin 2	actin binding and actin monomer binding
CCDC6	CCDC6	SH3 domain binding and structural constituent of cytoskeleton
AKAP10	A-Kinase Anchoring Protein 10	protein kinase A binding
NHLH2	Nescient Helix-Loop-Helix 2	protein dimerization activity and RNA polymerase II activating transcription factor binding
LDLR	Low Density Lipoprotein Receptor	calcium ion binding and low-density lipoprotein particle binding
SLC1A2	Solute Carrier Family 1 Member 2	L-glutamate transmembrane transporter activity and glutamate:sodium symporter activity
RIMBP2	RIMS Binding Protein 2	cell junction
SAMD8	Sterile Alpha Motif Domain Containing 8	transferase activity
ABCE1	ATP Binding Cassette Subfamily E Member 1	ATPase activity and ribonuclease inhibitor activity
KIAA1244	ARFGEF Family Member 3	ARF guanyl-nucleotide exchange factor activity
TTBK1	Tau Tubulin Kinase 1	transferase activity, transferring phosphorus-containing groups and protein tyrosine kinase activity
SLC38A2	Solute Carrier Family 38 Member 2	symporter activity and amino acid transmembrane transporter activity
MYH11	Myosin Heavy Chain 11	calmodulin binding and motor activity
EIF5A2	Eukaryotic Translation Initiation Factor 5A2	RNA binding and translation elongation factor activity
SUN2	Sad1 And UNC84 Domain Containing 2	identical protein binding and lamin binding
SLC35F3	Solute Carrier Family 35 Member F3	integral component of membrane
SNTB2	Syntrophin Beta 2	calmodulin binding

BEND4	BEN Domain Containing 4,	n.d
ZCCHC14	Zinc Finger CCHC-Type Containing 14	nucleic acid binding and phosphatidylinositol binding
UBE2D1	Ubiquitin Conjugating Enzyme E2 D1	ligase activity and acid-amino acid ligase activity
XPR1	Xenotropic And Polytropic Retrovirus Receptor 1	G-protein coupled receptor activity and transmembrane signaling receptor activity
PRKAA2	Protein Kinase AMP-Activated Catalytic Subunit Alpha 2	transferase activity, transferring phosphorus-containing groups and protein tyrosine kinase activity
MYLIP	Myosin Regulatory Light Chain Interacting Protein	ligase activity and ubiquitin protein ligase activity
RMND5A	Required For Meiotic Nuclear Division 5 Homolog A	GID complex
LRP6	LDL Receptor Related Protein 6	protein homodimerization activityand signaling receptor binding
ATP6V1B2	ATPase H+ Transporting V1 Subunit B2	proton transmembrane transporter activity
NUS1	Dehydrodolichyl Diphosphate Synthase Subunit	transferase activity, transferring alkyl or aryl (other than methyl) groups
USP6	Ubiquitin Specific Peptidase 6	nucleic acid binding and cysteine-type endopeptidase activity.
PLCB1	Phospholipase C Beta 1	calcium ion binding and enzyme binding
LCLAT1	Lysocardiolipin Acyltransferase 1	transferase activity, transferring acyl groups and 1-acylglycerol-3-phosphate O-acyltransferase activity
TNRC6A	Trinucleotide Repeat Containing 6A	nucleic acid binding and nucleotide binding
SLC7A5	Solute Carrier Family 7 Member 5	peptide antigen binding and antiporter activity
STIM2	Stromal Interaction Molecule 2	calcium ion binding and store-operated calcium channel activity
KREMEN1	Kringle Containing Transmembrane Protein 1	integral component of membrane
CFL2	Cofilin 2	actin binding
EPG5	Ectopic P-Granules Autophagy Protein 5 Homolog	cytoplasm
PHACTR2	Phosphatase And Actin Regulator	actin binding and protein phosphatase inhibitor activity
TBL1XR1	Transducin Beta Like 1 X-Linked Receptor 1	transcription regulatory region DNA binding and histone binding
CPEB4	Cytoplasmic Polyadenylation Element Binding Protein 4	nucleic acid binding and RNA binding
ACVR1	Activin A Receptor Type 1	protein homodimerization activity and protein kinase activity
SIX4	SIX Homeobox 4	DNA binding transcription factor activity and sequence-specific DNA binding
HPRT1	Hypoxanthine Phosphoribosyltransferase 1	protein homodimerization activity and nucleotide binding
NFE2L1	Nuclear Factor, Erythroid 2 Like 1	DNA binding transcription factor activity and transcription coregulator activity
IKZF4	IKAROS Family Zinc Finger 4	nucleic acid binding and protein homodimerization activity
TNFRSF10B	TNF Receptor Superfamily Member 10b	TRAIL binding
TMEM170B	Transmembrane Protein 170B	plasma membrane
ZFYVE26	Zinc Finger FYVE-Type Containing 26	phosphatidylinositol-3-phosphate binding
FAM43A	Family With Sequence Similarity 43 Member A	n.d.
ZEB2	Zinc Finger E-Box Binding Homeobox 2	nucleic acid binding and phosphatase regulator activity
USP48	Ubiquitin Specific Peptidase 48	cysteine-type endopeptidase activity and thiol-dependent ubiquitinyl hydrolase activity
MBNL3	Muscleblind Like Splicing Regulator 3	nucleus and cytoplasm
GABRA4	Gamma-Aminobutyric Acid Type A Receptor Alpha4 Subunit	chloride channel activity and GABA-A receptor activity
SKIDA1	SKI/DACH Domain Containing 1	n.d.
NIPA1	NIPA Magnesium Transporter 1	magnesium ion transmembrane transporter activity
SERPINE1	Serpin Family E Member 1	signaling receptor binding and protease binding
BRWD3	Bromodomain And WD Repeat Domain Containing 3	nucleus
C3orf58	Chromosome 3 Open Reading Frame 58	golgi membrane
CBY1	Chibby Family Member 1	identical protein binding and beta-catenin binding
PTGFRN	Prostaglandin F2 Receptor Inhibitor	protein binding
DGKE	Diacylglycerol Kinase Epsilon	NAD+ kinase activity and diacylglycerol kinase activity
ATXN1	Ataxin 1	identical protein binding and chromatin binding
RASA1	RAS P21 Protein Activator 1	signaling receptor binding
BTBD7	BTB Domain Containing 7	nucleus
KLHL42	Kelch Like Family Member 42	ubiquitin-protein transferase activity
CCND2	Cyclin D2	protein kinase binding
DLC1	Rho GTPase Activating Protein	GTPase activator activity and SH2 domain binding
FZD3	Frizzled Class Receptor 3	G-protein coupled receptor activity and PDZ domain binding
DLG5	Discs Large MAGUK Scaffold Protein 5	beta-catenin binding and receptor signaling complex scaffold activity
PTP4A1	Protein Tyrosine Phosphatase Type IVA, Member 1	phosphatase activity and protein tyrosine/serine/threonine phosphatase activity
ELL2	Elongation Factor For RNA Polymerase II 2	protein binding
C16orf70	Chromosome 16 Open Reading Frame 70	protein binding
UCP3	Uncoupling Protein 3	transporter activity and oxidative phosphorylation uncoupler activity
STX16	Syntaxin 16	syntaxin binding and SNAP receptor activity
PLA2G12A	Phospholipase A2 Group XIIA	calcium ion binding and calcium-dependent phospholipase A2 activity
B4GALT5	Beta-1,4-Galactosyltransferase 5	galactosyltransferase activity
TRPS1	Transcriptional Repressor GATA Binding 1	DNA binding transcription factor activity and sequence-specific DNA binding
ONECUT2	One Cut Homeobox 2	DNA binding transcription factor activity and transcriptional activator activity, RNA polymerase II proximal promoter sequence-specific DNA binding
UBE2D2	Ubiquitin Conjugating Enzyme E2 D2	ligase activity and acid-amino acid ligase activity
TAOK1	TAO Kinase 1	transferase activity, transferring phosphorus-containing groups and protein tyrosine kinase activity
MSANTD4	Myb/SANT DNA Binding Domain Containing 4	nucleus
MIB1	Mindbomb E3 Ubiquitin Protein Ligase 1	ligase activity and ubiquitin-protein transferase activity
SCN9A	Sodium Voltage-Gated Channel Alpha Subunit 9	ion channel activity and sodium ion binding
BRWD1	Bromodomain And WD Repeat Domain Containing 1	protein binding

4.5. Senescence analysis

We hypothesized that a hypocaloric MD together with PA promotes a better aging profile in late adulthood, and I showed that the intervention is associated to a decrease in miR-130a and miR-30c. Both microRNAs have been associated with aging before (276, 277).

Then, we analyzed monocytes senescence. For this purpose, we first measured *CD28* and *CD57* gene expression. It has been reported that CD28 expression is reduced (278) and CD57 is increased in senescent monocytes (279). We observed that *CD28* levels are higher (p = 0.0709 and that CD57 levels are lower in the intervention group that control group. Although these results are not significant, probably because of the small size of the population, they suggest that intervention is delaying monocyte senescence. **(figure 41)**.

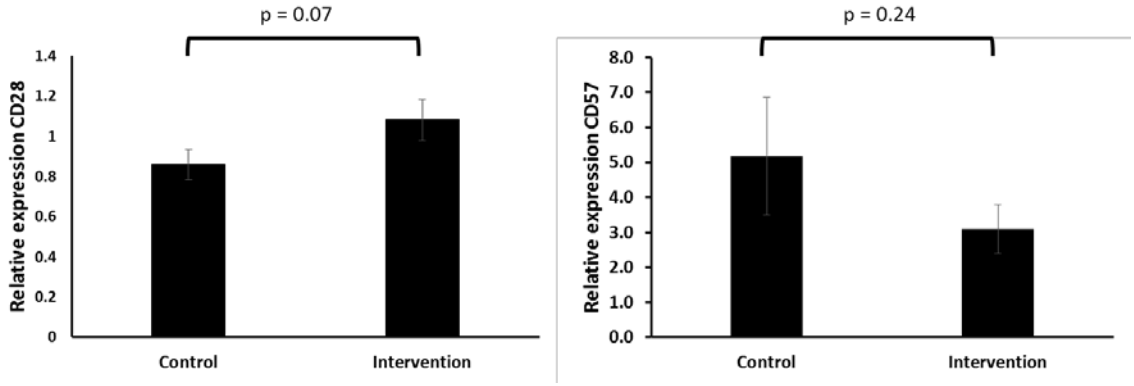

Figure 41: Changes in relative expression of *CD28* and *CD57* in monocytes after 1 year of intervention. p value refers to the intra-subject's comparison of the paired-repeated measures t-student test. Data represent mean ± SEM

DISCUSSION

It is well known that food intake is one of the most important environmental factors affecting human health. Nutrients are needed to maintain cellular processes. But nutrients are not only mere energy providers, they are also key regulators of gene expression and they interact with our genome to modulate a plethora of biological processes (280). For this reason, understanding how human being could be affected by specific foods or dietary patterns, specifically in relation to gene expression, has a key role in nutrition research field. In the last years, epigenomic has emerged as an interesting mechanism of gene expression regulation with a great potential to be used as therapeutic target. Not only nutrients interact with our genome, they also do that with the epigenome. MicroRNAs have been recently unraveled as important fine-tune modulators of gene expression. Previous studies in cell and animal models have shown how different fatty acids modulate the expression of different microRNAs (178, 179). This thesis aimed to describe how Mediterranean foods and the MD pattern modulate the expression of microRNAs in human interventional trials.

1. Extraction and detection of exogenous microRNAs

One of the most intriguing result of the last decade is the detection of plant microRNAs in human plasma (246). We aimed to elucidate if Mediterranean foods also provided microRNAs as new micronutrients. We analyzed the presence of microRNAs in beer and EVOO samples. According to our bioanalyzer results, we extracted microRNAs from beer and EVOO, although at a very low concentration that could be due to the processed nature of these materials. A previous study failed to detect substantial levels of microRNAs in fruits usually consumed by humans (281). Besides, olive microRNAs have been described before from growing and dormant lateral bud samples using NGS (269). The discordance between Donaire and colleagues (269) results and ours could be due to the different sample used. Instead of leaves, roots or the olive fruit, we used EVOO, a mechanically processed product from olives. Additionally, EVOO is an extremely fatty liquid. Although we introduced modifications in the protocol to cope with this limitation, some technical caveats could be influencing our yielded RNA. On the other hand, beer is also a processed liquid submitted to a fermentation process that generates alcohol. It has been described that plant microRNAs are resistant to different conditions that contribute to nucleic acid degradation, including heat, acid pH and enzymatic lysis (246).However it is possible that microRNAs from the olive oil and beer were degraded in such fatty compound and in the fermentation process of beer production, respectively. However, our spike-in analysis showed that a plant single-strand microRNA exogenously added to the sample could be detected by RT-qPCR suggesting that, although some technical limitations exist, the technique is sensitive enough to measure plant microRNAs.

We then analyzed the presence of plant microRNAs in the plasma of healthy volunteers after intake of 40 ml of EVOO. Interestingly, we found 109,295 molecules of the miR-1529 belonging to soybean. However, all the read sequences presented missing nucleotides when comparing with the canonical sequence and 3 mismatches **(table 9)**. We also failed to identify any of the sequences reported by Donaire and colleagues (269). These results do not support the results reported by Zhang *et al*, although we used healthy volunteers that usually consume EVOO.

On the other hand, the results obtained by Zhang *et al* are very controversial due to a great number of papers supporting and refusing the hypothesis of these authors. For instance, Dickinson B. *et al* tried to replicate the experiments without success (251). On the other hand, some other authors support this hypothesis. In ghtis regard, Chin AR *et al* that detect plant miR-159 in human serum (249). However, the number of copies was very low and when they tried to replicate the experiment in cell and animal models the amount of mimic miR-159 that they proportionated was not physiological. It is better established in the case of microRNA transference from breast milk to breast milk-fed infants (282). In other animals models as pigs, who were fed with 1 kg of maize, Yi L. *et al* detected maize-microRNAs in pig serum (283). In the case of miR-451 KO mice model Wang W. *et al* detected dietary miR-451 in blood of these animals (284). Other authors refused this hypothesis due to the degradation and low bioavailability of dietary microRNAs (285). In our study, we supposed that the levels of EVOO microRNAs in the plasma of our volunteers would reach a peak after 2 h. Baier *et al* (286) showed a maximum increased of milk-derived microRNAs between 4 and 8 h post-ingestion, although a significant increase could also be observed after 2 h with the maximum dose. However, Zhang *et al* (246) detected exogenous plant microRNAs in overnight-fasted animals. We analyzed the plasma of five volunteers that usually consume EVOO, so we expected to detect EVOO microRNAs, even if after 2 h of the EVOO ingestion the levels of EVOO microRNAs did not reached their peak. We must bear in mind that although NGS using Ion Torrent technology offers a high microRNA detection rate that is really useful for challenging samples like EVOO, such exceptional high sensitivity may be partly related to library contamination and should be interpreted cautiously (287-289). Thus, we cannot rule out the possibility that the detection of plant microRNAs in EVOO and plasma samples was the result of a technical artifact, likely due to a library contamination. Thus, despite our strategies to maximize the presence of EVOO microRNAs in the plasma of the volunteers we could not confidently detect them.

In summary, our results contribute to the current controversy about the presence and the dietary uptake of plant microRNAs by adding experimental evidences that suggest that plant-based processed products, like beer and EVOO do not contain microRNAs or they are not consumed through the diet. Specifically, we did not detect substantial quantities of plant

microRNAs in the EVOO and beer samples. Furthermore, we did not detect plant microRNAs in the human plasma after the ingestion of an acute dose of EVOO.

2. VOHF study

EVOO intake, the main fat source in MD, is well described as a beneficial food with multiple effects, including anti-oxidant, anti-inflammatory and anti-microbial activities (88). Additionally, results from the VOHF study have shown a dose-dependent effect on HDL proteome (81). However, the effect of OO on gene regulation has not been well described so fare. Nutrigenomics studies related to OO are mainly focused in the effect of its bioactive compounds, as hydroxytyrosol (83) or oleic acid (290) although there are a few studies about the nutrigenomic effect of OO (90, 290). Other studies have explored the effect of an acute ingestion of VOO in gene expression of health volunteers (90). Authors observed differences in insulin-related genes such as O-linked-N-acetylglucosamine transferase (*OGT*) and arachidonate-5-lipoxygenase-activating protein (*ALOX5AP*) (90). Other study showed the effect of VOO combined with MD in healthy volunteers during 3 months in gene expression related to inflammation as INF-gamma (*INFgamma*), Rho GTPase-activating protein15 (*ARHGAP15*), and interleukin-7 receptor (*IL7R*) or oxidative stress such as adrenergic as beta (2)-receptor (*ADRB2*) and polymerase (DNA-directed) kappa (*POLK*) (91). All these studies point towards and anti-inflammatory and cardioprotective impact of OO on gene expression.
Such impact could be mediated by an effect of OO on some related microRNAs. However, studies focusing on the analyses of epigenetic regulation mediated by VOO intake still lack.

2.1 Circulating microRNAs regulation with L-EVOO intake

We observed differences in the regulation of n microRNA expression depending of polyphenol enrichment of each type of VOO. In L-EVOO (EVOO supplemented with 250 mg/Kg of its own polyphenols) let-7e, miR-17, miR-20 and miR-328 were differently modulated **(figure 15)**. Let-7e expression showed a bimodal distribution with two downregulation peaks at 1 hour and 4 hours (being non-significant at 4 hours), suggesting a short-term effect at 1 hour and a mid-term effect at 4 hours. Let7-e is a well-known microRNA, involved in many processes, like IGF-1 regulation (168), and inflammation, where has been reported that its upregulation inhibited inflammatory responses (291) , a recognized effect of VOO ingestion (292). Circulating let-7e has been found strongly dysregulated in atherosclerotic disease (293) and its levels are higher in patients with ischemic stroke (294). Moreover, in hypertensive patients, let-7e was also found upregulated comparing to healthy volunteers (295) and let-7e levels has been negatively correlated with HDL

levels and positively correlated with the number of metabolic syndrome criteria in school-age children (296) . Thus, these results suggest that a downregulation of let-7e could be a positive effect of EVOO intake and that a mechanism whereby EVOO has this effect could be the regulation of this microRNA.

After L-EVOO intake, miR-17 and miR-20a levels were increased at all time-points, being significant at 4 hours in miR-17 and at 1 and 2 hours in miR-20a. Both microRNAs showed the same tendency, as expected, due to the fact that they belong to the same cluster (miR-17-92 cluster). This cluster is related to IGF1/PI3K/AKT/mTOR and AMPK/SIRT1/PGC1α pathways (217, 218). MiR-17 overexpression leads to the activation of AKT/mTOR, promoting cell adhesion, migration and proliferation (297); and expression of the miR-17-92 cluster impairs AMPK activation in mantle cell lymphoma (MCL) cells (298). Moreover, miR-17-92 cluster is highly expressed in endothelial cells (299) and the overexpression of miR-92a in human umbilical vein endothelial cells (HUVECs) and mice impaired angiogenesis *in vivo* and *in vitro* (300). In addition, the reduction of miR-17-92 cluster expression has also been observed to result in more ROS and DNA damage, which increases age-related damage (219). Interestingly, the levels of circulating miR-17-92 cluster were significantly reduced in coronary artery disease patients compared with healthy controls (301). A reduction in circulating levels of miR-20a has also been found in South African Women with Gestational Diabetes Mellitus (GDM) (302). Moreover, circulating miR-20a has been also linked to aging as it was found more abundant in young donors comparing to old donors after a resistance exercise in male volunteers (303). On the other hand, Ye D. *et al* observed that levels of miR-17 and miR-20a were upregulated in T2DM patients with non-alcoholic fatty liver disease (NAFLD). In addition, GLUT4, a glucose transporter with a key role in peripheral tissues glucose uptake, is a direct target of miR-17 and a critical regulator of glucose metabolism (304). Therefore, the role of miR-17-92 cluster in human health is controversial with studies showing a benefit derived from its overexpression and studies showing a detrimental effect. In summary, the increase in miR-17-92 cluster levels could protect against ROS and DNA damage and could promote healthy aging through this protection. However, an increase in the levels of microRNAs from this cluster could also promote the development of T2D. It is likely that the effect of miR-17-92 cluster could be different in different tissues, in response to different environmental exposures or in different ages. More studies are needed to clarify if non-enriched EVOO also increases circulating levels of these microRNAs and if this L-EVOO mediated increase is related to a better aging profile or to a poorer glucose control. It would be notice that the studies carried out by Keita H *et al.* showed an insulin resistance in mice fed a high fat diet based in EVOO (305), but, in human intervention studies, an improvement in insulin resistance with EVOO intake has been observed (306).

Finally, miR-328 is repressed at 1 hour and its levels increase after then to reach back to basal levels **(figure 15)**. Increased levels of miR-328 have been suggested to be predictive of AMI (307) and are associated to AF, even after the adjustment for risk factors like hypertension. This miRNA has been also found to promote atrial electrical remodeling by reducing L-type Ca (2+) channel density (308). These results suggest that the reduction of circulating miR-328 levels by L-EVOO could have a beneficial effect on CVD.

As it has been described before, an over expression of IGF-1/PI3K/AKT/FOXO/mTOR pathway is related to aging. Additionally, higher levels of plasma IGF-1 and lower activity of the IGF1R are negatively associated with longevity (138), whereas lower levels of plasma IGF-1 were associated with greater survival in nonagenarian women (139). Our *in* silico analysis have shown that these microRNAs could regulate mechanisms associated to insulin sensitivization via IGF1 and IGF1R regulation. On the other hand, greater longevity has also been observed in populations with high adherence to the MD (73, 74) for which the main fat is EVOO. The modulation of let-7e, miR-17, miR-20 and miR-328 by L-EVOO could explain the protective effect of EVOO regarding ROS damage and endothelial function that could be part of the mechanism whereby MD increased longevity.

2.2. Circulating microRNAs regulation with M-EVOO intake

MiR-17 and let-7e were also modulated by M-EVOO. Let-7e showed the same bimodal response to M-EVOO than observed with L-EVOO. This suggest that this microRNAs is a primary target of enriched EVOO and deserves further consideration to elucidate the mechanisms behind the EVOO-mediated modulation of let-7e. miR-17 and miR-20a levels also responded similarly to L-EVOO as they were also increased by M-EVOO, although the increase in miR-20a was not significant. Again, these results point to the miR-17-92 cluster as one of the main targets of EVOO intake and one of the epigenetic mechanisms by which EVOO intake modulate cellular physiology. Moreover, these results suggest that miR-17 and miR-20a could be potential biomarkers of EVOO intake, although, we did not observe a dose-dependent response in relation to the higher polyphenol content of M-EVOO comparing to L-EVOO. Further studies with larger sample sizes are needed to confirm the modulation of the miR-17-92 cluster by EVOO and the impact of such modulation. On the other hand, we observed that miR-192 was upregulated at 6 hours by M-EVOO. Circulating miR-192 levels have been suggested as potential biomarker of ischemic cardiomyopathy (ICM) survival. The median age at time of death was 84 years in patients with low miR-192 expression but 67 years with high miR-192 expression. However, miR-192 levels in non-ICM patients is not associated with survival (309). Another study suggested that increased levels of circulating miR-192 might be a conserved biomarker for obesity or metabolic state in a murine model where authors observed an increase in circulating

miR-192 levels in response to a high-fat / high-fructose diet (310). The role of miR-192 in cancer has been deeply studied, but little is known about its role in CVD, diabetes or aging and most studies have been carried out in animal models or in individuals with CVD, while our study was carried in healthy volunteers. Therefore, it should be defined if an increase in circulating miR-192 levels in healthy individuals have a short-term beneficial effect. But, for that purpose, a new study designed to directly asses that hypothesis is needed.

2.3. Circulating microRNAs regulation with H-EVOO intake

Interestingly, let-7e was also downregulated by H-EVOO following a bimodal distribution (**figure 17**), although an inverse response was observed when comparing with L-EVOO and M-EVOO (**figure 21**). This point to let-7e as the most consistently modified microRNA by EVOO intake and suggest it is a good biomarker of EVOO intake and a potential mediator of the effects of EVOO.

We also observed a down regulation of miR-10a with a greater effect at 2 hours **(figure 17).** It has been shown that miR-10a represses the expression of proteins that destabilize IκB/NF-κB–mediated inflammation in cultured human aortic endothelial cells (HAEC) (311). Simionescu N. *et al* observed that circulating miR-10a was upregulated in hyperlipidemic patients and it is positively correlated with serum lipids and inflammatory parameters (312). Thus, a decrease in miR-10a levels mediated by H-EVOO intake could explain the beneficial effect on lipid levels and inflammation attributed to EVOO and its polyphenols (313-315).

MiR-21 and miR-26b were also downregulated with a minimum at 2 hours **(figure 17)**. miR-21 ablation in macrophages of atherogenic mice has been found to increase apoptosis, plaque necrosis, and vascular inflammation, promoting plaque instability and atherosclerosis progression (182). However, circulating miR-21 has been found downregulated after an acute exercise in basketball athletes (198). Moreover, miR-21 levels have been related to aging, specifically, lower levels of miR-21 are related to healthy aging and it has been postulated as a biomarker of healthy aging (316, 317). These results suggest that H-EVOO mediated modulation of miR-21 could link EVOO intake and healthy aging, although high levels of polyphenols are needed to modulate this microRNAs as it did not change with L-EVOO and M-EVOO. In this regard, a further analysis of the link between miR-21, EVOO and aging is guaranteed.

Finally, miR-26b is decreased in cardiac death or recurrent myocardial infarction, but it has been also shown that its low levels are associated to the prevention of adverse cardiomyocyte hypertrophy (318) and it has been observed that its overexpression produces enhanced endothelial cells proliferation, migration, and tube formation and its downregulation suppressed the proliferative and angiogenic capacity of endothelial cells (319). On the other hand, levels of circulating miR-26b of hypertensive patients with left ventricular hypertrophy

(LVH) are higher compared to healthy subjects (320). It should be studied the effect of the H-EVOO mediated downregulation of miR-26b on cardiovascular health in more detail.

2.4. Functional analyses of microRNAs modulated by the enriched EVOOs

The functional analysis shed some interesting results. For instance, *PRKCB*, a gene involved in endothelial cell proliferation, intestinal sugar absorption, insulin signalling and B-cell activation (270) and *SCD* (Stearoyl-CoA Desaturase), involved in MUFA synthesis were targets of all 4 L-EVOO modulated microRNAs (**figure 18, table 9**). Also, between the common genes of let7-e and miR-328, two micro-RNAs with the same response to L-EVOO, we found *STARD7* (*StAR related lipid transfer domain containing 7*) that mediates intracellular trafficking of lipids (321) and induces ROS production and reticulum endoplasmic stress (322), and *IGF1R,* a key regulator of insulin signaling pathway (138). It would be interesting to further study the link between L-EVOO, the modulated microRNAs and the expression of *PRKCB*, *SCD, STARD7* and *IGF1R*. Further studies are needed to elucidate the mechanisms behind such link, but these results are a primary mechanistic glance and suggest that fatty acid metabolism and insulin signalling could be directly affected by this link.

In silico studies showed that M-EVOO modulated microRNAs were involved in cancer but also in insulin pathways (**figure 19, table 10**). Twenty-five genes are common targets of the 3 microRNAs differentially expressed. Among them, there is *PRKAA2*, the catalytic subunit of the AMPK cellular energetic sensor. This result suggest that M-EVOO could be modulating the energetic status of the cell through the microRNA-mediated regulation of this gene. Such a modulation could have an impact in aging as it has been shown that AMPK increase is associated with longevity in animal models (323). Moreover, metformin, an antidiabetic drug that targets AMPK has been studied because of its effect on longevity. Nevertheless, this interesting suggestion need to be experimentally confirmed.

In silico analyses of microRNAs modulated by H-EVOO showed that they are mainly involved in cancer, but also in circadian rhythm and in one of the nutrient sensing pathways studied, PI3K/AKT pathway (**figure 20, 21 and table 11)**. Only 1 gen is commonly regulated by the 4 microRNAs, *YOD1.* This gene is a protein deubiquitinase involved in inflammation (271). The fact that this gene is a potential target of all common microRNAs modulated by H-EVOO suggest that the effect of this highly enriched EVOO on health could be mediated primarily by *YOD1* modification. Nevertheless, the direct targeting of *YOD1* be the 4 microRNAs should be experimentally validated as well as the link between H-EVOO, the 4 microRNAs and *YOD1*.

Finally, we searched for common targets of the microRNAs modulated by all three oils (**figure 21**). Forty-four genes were targeted by common microRNAs. Among them, we found *ABCG2, PRKAA2* (Protein Kinase AMP-Activated Catalytic Subunit Alpha 2) or *SCD,* all genes regulated by

diet (324-326). The finding of *PRKAA2* as a gene targeted by common microRNAs should be highlighted given its link to one of the aging-related nutrient sensing pathways, AMPK, described above. These results could suggest that the mechanism by which these microRNAs regulates AMPK activity could be through *PRKAA2*.

In summary, we observed that enriched EVOO consistently modified microRNAs that target genes related to insulin signalling pathways as well as lipid metabolism and inflammation. Among the nutrient sensor pathways, AMPK pathway may be the most affected by all three enriched EVOO. These results suggest that, providing that EVOO impact on human longevity, AMPK modification could be the underneath mechanism. In this regard, further studies to analyse this hypothesis are guaranteed.

It is important to mention that microRNAs of the miR-17-92 cluster as well as let-7e are commonly modified by the different types of functional EVOOs, although we did not find a clear dose-dependent response. That suggests that these microRNAs could play a key role in the mechanisms by which EVOO exerts its protective effects, but that polyphenol supplementation could not have an additive effect. However, in previous studies with the VOHF intervention, authors detected differences between L-EVOO, M-EVOO and H-EVOO regarding the levels of plasma phenolic metabolites (327), enzymes related to HDL antioxidant activity, oxidative stress, lipid profile, fat soluble vitamins or glucose and insulin resistance (255, 328) . Our results suggest that a different concentration of polyphenols don't affect circulating microRNAs expression. On the other hand, Pedret A *et al* did not observed changes in inflammation biomarkers depending on phenolic composition of functional EVOOs (328). Moreover, our results showed more similarity between L-EVOO and M-EVOO than with H-EVOO regarding circulating microRNAs. The explanation could be that the low or medium enrichment saturate circulating levels of these microRNAs. Could also be possible that a higher polyphenol enrichment had different effects on molecular pathways and a different impact on human aging. In this regard, it has been shown that an excess of polyphenols could produce pro-inflammatory effects (329). It has been also described that an excess of flavonoids could be associated with an oxidant effect (330). These results suggest that the different effect of H-EVOO on microRNA levels could be, in part, due to a detrimental effect of an excess of polyphenols and, thus, the polyphenol supplementation should be uses carefully.

2.5. Study limitations and future perspectives

This study has some limitations. First, the low number of participants in the study with only 12 volunteers. In addition, not all volunteers completed the intervention, reducing the total number of participants. We should also bear in mind that it is a postprandial study and some changes produced by EVOO intake could need a longer exposure. Moreover, participants drunk

30 ml of EVOO that is a dose which is not usually consumed in only one ingestion. It would be necessary to carry out another intervention study with regular doses of EVOO ingested in a regular basis (for example in one day). Furthermore, it should be analysed the effect of each specific polyphenol in every concentration on the expression of the modulated microRNA to discern if EVOO oil composition have a specific effect in microRNA regulation. Finally, some technical limitations linked to the detection and normalization of microRNAs in plasma could interfere with the results by reducing sensitivity.

Nonetheless, this study depicts a possible mechanism that could explain the beneficial effect of EVOO by the consistent modulation of let-7e, and miR-17-92 cluster, microRNAs that are well described because of their influence in insulin signalling pathways and inflammation and deserve further attention. Moreover, miR-17-92 cluster downregulation has been consistently described as biomarker of aging in several models of aging (331), so its upregulation produced by EVOO intake could mediate an antiaging effect, but further studies to confirm such effect such hypothesis are needed. .

3. MiRoBeer study

3.1 Analysis of polyphenols levels in plasma and urine

First, we analyzed the presence of IX and 8-PG in plasma and urine of volunteers. Both are beer derived metabolites and IX has been shown to be a good marker of beer consumption (275). We aimed to confirm participants' adherence to the intervention. This analysis was carried out with the collaboration of Dr. Ana Rodríguez Mateos through a 3-months internship in her laboratory, in the Division of Diabetes and Nutritional Sciences of King´s College London. First, we measured the concentration of both metabolites in beer and non-alcoholic beer and, as expected, beer presents high levels of IX, higher than non-alcoholic beer; while levels of 8-PG are higher in non-alcoholic beer (**figure 22**). The possible reason for that could be the heat treatment applied to beer to dealcoholize it that favors the conversion of IX on 8-PG. In fact, this process is commonly produced in human intestine after beer intake by microbiota (332). Then, we measured them in plasma and urine of the participants in the study. We observed higher levels of IX after every intervention in plasma and urine, although it was significant only in plasma after beer intervention **(figure 23).** The increase in IX levels after beer intake suggest that participants adhered well to the intervention. The lack of validation of IX increase after non-alcoholic beer consumption is explained by the fact that IX concentration was low in non-alcoholic beer (**figure** 22) but does not indicate a lack of adherence to the intervention. However, 8-PG levels did not increase in urine or plasma after consumption of non-alcoholic beer. One reason for that could be that 8-PG is a secondary metabolite of IX and have a longer half-life than IX so part of the IX

could be transformed in 8-PG but with intrinsic differences between patients given that this conversion depends of factors like the microbiota of each individual (332). In fact, different conversion phenotypes have been described and this could be an explanation of the great variability that we detect in levels of 8-PG (333). Additionally, we allowed patients to choose the moment of the day of beer intake to avoid problems associated to alcohol and activities like driving, working, etc. Participants could also choose if they drunk the 500 ml of beer in 1 or more intakes. Martinez SE *et al* studied the enantiospecific pharmacokinetics of IX and 8-PF in rats concluding that the rate of urinary excretion is higher the closer the time of the intake (334). Given that we took blood and urine samples early in the morning, after 12 hours fasting, IX and 8-PG levels in urine or plasma could be lower than expected. The time since the last intake of beer could be delayed depending if the participant drunk the beer in midday or night, introducing a big variability in our urine and plasma measures.

3.2 Analysis of population characteristics

First, we analyzed if participants had modified their nutritional behavior during the intervention in order to control for that effect. We did not find any significant modification in anthropometric or body composition. We found some modifications in nutritional behavior such as a lower energy intake during intervention phases that was explained by a lower intake of all macronutrients (carbohydrates, proteins and fats), but only the decrease in MUFA intake was significant (**table 12**). This could be due to the so-called "control effect" that means that participants feel under supervision during intervention and, unconscienced, they improved their diet. Nevertheless, we considered that those weak modifications, non-significant, would not significantly affect circulating and macrophage microRNAs levels. Although changes in biochemistry parameters were not significant, we observed a tendency to reduce C reactive protein (CRP) levels, a classic inflammation biomarker (335) after the beer intake that continued lower along the whole intervention (**table 12**). We also observed a trend to a reduction in total cholesterol, LDL-cholesterol and HDL-cholesterol levels and an increase in triglycerides after each intervention period (**table 12)**. It has been described the influence of beer consumption in inflammation. In animal model, Iso-α-acids, a component of beer produces a significant reduction of inflammatory cytokines like IL-1β (336). In vascular cells, IX and XN manifested anti-inflammatory properties while 8-PG increase blood vessels formation (337). In healthy male runners, non-alcoholic beer reduces levels of IL-6 (338), while in elderly women whose diet were supplemented with 500 ml of non-alcoholic beer, inflammatory parameters did not change while oxidative stress parameters improved although only in participants with cholesterol levels higher than 240 mg/dl a significant reduction was found (339). Moreover, recent studies conducted by Padro T. *et al* observed that beer consumption did not produce an improvement

in anthropometric parameter as well as lipoproteins profile or inflammation biomarker while anti-oxidative properties of HDL and better cholesterol efflux were detected (340). These results agreed with our results given that we did not observed significant differences in lipid levels after each intervention period, but we observed a tendency to decreased CRP levels.

3.3.Modulation of circulating microRNAs after beer intake

Some circulating microRNAs modified by beer intake are common with EVOO. For instance, miR-328, which was downregulated by L-EVOO, was also downregulated by non-alcoholic beer. It was up-regulated by beer, although not significantly and the difference in plasma miR-328 levels among beer and non-alcoholic beer was significant. This suggest that the alcohol content of beer could damper miR-328 modifications mediated by polyphenols of the beer and the EVOO. As it has been described before, miR-328 is a microRNA whose higher levels are correlated with CVD (307). Therefore, while non-alcoholic beer could be promoting a beneficial effect related to miR-328, alcoholic beer could have the opposite effect. A similar response was found in miR-92a, (**figure 25**). In osteosarcoma patients, miR-92a regulated the expression of PTEN/AKT signaling pathway (341) and an inhibition of miR-92a is associated to a better remodeling after myocardial infarction (342). MiR-92a is a member of the miR-17-92 cluster and, as it has been described before, the downregulation of this cluster could be a biomarker of healthy aging (331). MiR-17-92 cluster is closely linked to nutrient sensing pathways regulation (especially insulin signaling cascade) and inflammation (217-219). Moreover, in *Rhesus* monkeys a downregulation of miR-92 was observed with CR, a well-known intervention to promote longevity, although miR-92 levels were dependent on bodyweight or adiposity (343). Mir-17 levels were also downregulated with non-alcoholic beer, but also with alcoholic beer (**figure 27**). These results point again to the cluster miR-17-92 as one important mediator of the effects of traditional Mediterranean foods like EVOO and beer and could be the link between these foods and aging. In summary, members of the miR-17-92 cluster as well as miR-328 are sensitive to nutrients and could be good biomarkers of food intake.

Other circulating microRNAs modified by beer intake are miR-155 and miR-320. Regarding miR-155, it is upregulated by beer and downregulated by non-alcoholic beer, being the difference between both significant. Circulating levels of this microRNA have been found downregulated in esophageal cancer (344) and in diabetic patients (345). Moreover, Elgheznawy A *et al* observed lower levels of platelet miR-155 in humans and mice with T2DM (346). In addition to this, low levels of circulating miR-155 are associated to coronary artery disease (CAD) and has been suggested to be a potential risk factor of coronary heart disease (CHD). All these results could suggest that low levels of this microRNA could not be beneficial in the context of CVD and T2DM. More studies are needed to clarify the role of the biological impact of the non-alcoholic

beer-mediated downregulation of miR-155. Interestingly, the increase of miR-155 with beer could suggest that beer could affect cardiovascular health specifically through the modulation of this microRNA, as the effect of beer on the other microRNAs suggested a negative impact on human health. Finally, miR-320 was downregulated after both, beer non-alcoholic beer intervention, although it was significant only in the last condition. In plasma samples of diabetics patients, this microRNA was found downregulated (347). On the other hand, circulating miR-320 levels are higher in Crohn's disease (CD) (348) and an overexpression of miR-320 is related to cardiomyocyte apoptosis and the loss of mitochondrial membrane potential in mice (349). Additionally, overexpression of miR-320 in cardiomyocytes isolated from 2 months old Sprague-Dawley rats enhanced cardiomyocyte death and apoptosis while miR-320 KO rats showed the opposite effect (350). These results could suggest that the downregulation of miR-320 after non-alcoholic beer intervention could have a cardioprotective effect although in glucose metabolism could have a negative effect.

Finally, miR-107 is overexpressed almost significantly after each intervention. MiR-107 is related to insulin sensitivity in liver and its silencing improved glucose homeostasis and insulin sensitivity (351). As the results obtained in the other described microRNAs, this result agreed with a non-beneficial effect on glucose homeostasis after beer intake.

In silico functional analysis of the 4 circulating microRNAs differentially expressed (miR-92a, miR-328, mir-320 and miR-155) showed that only 4 genes are targets of all of them: *IGF1R, NUFIP2, MYO1D* (*myosin ID*) and *SOX11* (*SRY-Box 11*) (**figure 33a and table 13)**. *IGF1R*, the gene that encodes the receptor of the IGF1 is a key regulator of the insulin signaling pathway. *EEF2,* which is also regulated by 3 microRNAs (miR-155, miR-320, miR-92), is implicated in the regulation of protein kinase and could be regulated by diet, specifically, MD (352). The regulation of these genes could suggest that the microRNAs regulated by beer intake could have an effect on the insulin signaling pathway through the modulation of these genes. In the KEGG pathways analysis, some of the main pathways associated to genes targeted be these 4 microRNAs, are AMPK signalling pathway or PI3K-AKT signalling pathway, two nutrient sensing pathways with a role in aging **(figure 33b)**. *In silico* analysis showed the link between beer intake and regulation of IGF1/PI3K/AKT/mTOR pathway and AMPK/SIRT1/PGC1α pathway via miR-92a, miR-328, mir-320 and miR-155.

3.4. Modulation of macrophage microRNAs after beer intake

Macrophages microRNAs analysis after intervention showed that miR-17, miR-20a, miR-26b, miR-145 and miR-223 were differentially expressed **(figure 27).** Thus, we observed again the modulation of some members of the miR-17-92 cluster, miR-17 and miR-20a. This modulation in macrophages was similar to that found in plasma, they were upregulated by beer and downregulated by non-alcoholic beer. These results support this differential effect of beer and

non-alcoholic beer on this cluster. Both microRNAs play a key role in signal-regulatory protein α (*SIRPα*)-mediated macrophage inflammatory response (353). Moreover, in THP-1 and RAW 264.7 macrophage-derived foam cells lines, miR-20a decreased cholesterol efflux and increased its cholesterol content (354). Given that both are upregulated after beer intake but downregulated after non-alcoholic beer intake, this could suggest that alcohol could be mediating an inflammatory response in macrophages. However, the non-alcoholic beer-mediated decrease of both could have an anti-inflammatory effect. The recommendation for moderate alcoholic beer consumption is 2 drinks per day (355) but the current trend consider that the limit for alcohol consumption should be as maximum 10 g of alcohol consumption (356) On the other hand, miR-145, miR-26b and miR-223 showed a similar response to beer intake. It should be noted that miR-26b was also downregulated by H-EVOO, but not with the other less enriched EVOOs. As it has been described before, the reduction of its microRNA is associated to the prevention of adverse cardiomyocyte hypertrophy and its levels are higher in hypertensive patients with left ventricular hypertrophy (LVH) (318, 320). However, Zhang L. *et al* observed that an overexpression of miR-26b in bovine alveolar macrophages (bAMs) results in higher levels of pro-inflammatory cytokines and chemokines (357). This is consistent with an inflammatory effect of the alcohol of beer and an anti-inflammatory effect of non-alcoholic beer (358).

On the other hand, it has been shown that macrophage miR-145 expression is associated to inflammatory reaction via NF-κB activation in APOE KO mice (359). However, miR-145 was found downregulated in M1 macrophages comparing to M2 macrophages in bone marrow-derived macrophages (BMDMs) (360) and miR-145 expression inhibits the secretion of inflammatory factors with the downregulation of ADP ribosylation factor 6 (*ARF6*) in THP1 cell line (361). These results show controversial effects of miR-145 on macrophage inflammatory response and they seem to indicate that a downregulation of miR-145 could not be beneficial given that it could be associated to a pro-inflammatory profile in cell lines although in a mouse model of atherosclerosis the opposite could be true. Therefore, it would be necessary to carry out more studies to clarify the effect of miR-145 on humans.

The last macrophage microRNA differentially expressed is miR-223 that increased with beer and decreased with non-alcoholic beer. miR-223 is a key regulator of innate immunity and its expression could inhibit macrophage differentiation (362). Moreover, its downregulation increases TLR-activated inflammatory responses and promotes the production of pro-inflammatory cytokines IL-6 and IL-1β in murine model (363, 364). These results suggest that the modulation of miR-223 by non-alcoholic beer could promote inflammation, which is not in accordance with the effect of previously described microRNAs. It has been found an increase in miR-223 levels in serum from alcoholic patients and in serum and neutrophils in mice with

chronic alcohol consumption (365), which is consistent with our increase observed after beer intake. So, it is possible that the increase of miR-223 observed with beer was a specific effect of its alcohol content, whereas the relationship between the non-alcoholic mediated increase of miR-223 and its pro-inflammatory role should be further elucidated.

Finally, it is worth mentioning that let-7e, which was downregulated by all enriched EVOOs, was upregulated, almost significantly, by beer. As it has been described before, let-7e is involved in IGF-1 regulation (168). Upregulation of let-7e has been associated to stroke, atherosclerotic disease, hypertension and metabolic syndrome (293-296). These results could suggest that the beer-mediated increase of this microRNA in macrophages could be detrimental. This supposition needs to be directly addressed, because it has been reported that its overexpression in mice macrophages improve sensitivity and tolerance to lipopolysaccharides (LPS) in an AKT KO mouse (366). Upregulation of let-7e has also been associated to inhibited inflammatory responses in vascular smooth muscle cell (SMC) (291). Thus, let-7e could have tissue-specific effects and, in this sense, its increase in macrophages could be related, not to increase of CVD risk factors, but to an improvement in insulin sensitivity and a reduction in inflammation.

3.5.Macrophage and circulating microRNAs were correlated

Changes in circulating levels of let-7e, miR-328 and miR-26b and miR-92a correlated positively with changes in macrophage levels of these microRNAs (**figure 29**). This suggest that beer consumption has a similar effect in those microRNAs in macrophages and in their release to the circulation. We cannot assure that the origin of those microRNAs in the circulation is the macrophages, but such positive correlation suggest that this could be the case. It is well known that macrophages actively release microRNAs into the circulation (367). Thus, it is not unreasonable to suggest that macrophages could be, in part, the source of these plasma microRNAs.

Then, we performed correlation analyses to analyze If microRNA levels were associated to the characteristics of the individuals. Interestingly, we observed a positive correlation between plasma miR-320 and LDL cholesterol **(figure 31).** As far as we know, this is the first study reporting such correlation, although Wei G. *et al* previously observed a negative correlation between hepatic miR-320 and Adiponectin receptor 1 (ADIPOR1) protein, that promotes fatty acid oxidation in the liver (368). Interestingly, macrophage let-7e levels were negatively correlated with iron, soluble fiber and B1 vitamin intake **(figure 32)** It has also been demonstrated that a low-protein diet leads to a downregulation of plasma let-7e **i**n mice offspring, suggesting the influence of the diet on the expression of let-7e (369). To our knowledge, this is the first report showing a correlation between macrophage microRNA levels and dietary parameters. We cannot assure if the reduction observed in the intake of these

nutrients is influencing the response of let-7e to beer intake or if the effect of beer on let-7e levels is influencing the intake of these nutrients and new studies are needed to clarify these correlations.

The gene network analysis **(figure 35)** showed that there is a node formed *by CHD9, ZNF148, MEF2D, CSNK1A1, CNOT6L, HDAC4, TNRC6A, ABCA1* and *PPARα* as genes regulated by circulating microRNA modified by beer intake. They are mainly related to immune system function and glycolytic process and could be the mechanism by which beer intake impact on these processes. Future studies will be addressed to confirm the modification of the expression of those genes by beer intake. On the other hand, genes targeted by microRNAs modulated in macrophages were distributed in two different nodes involved in immune system function and nutrient sensing pathways. Given that nutrient sensing pathways are related to aging, the microRNA mediated modulation of those genes by beer could be a link between beer intake and healthy aging.

To sum up, we demonstrated that beer intake modulates the microRNA profile in both plasma and macrophages. Some of the modulated microRNAs had been found also modulated by the enriched EVOOs (let-7e, miR-328 and the miR-17-92 cluster), suggesting that they are specifically sensitive to nutrient-mediated modulation. Moreover, we observed a difference regarding beer and non-alcoholic beer intake with beer having a more pro-inflammatory microRNA profile that is inversed in non-alcoholic beer. This is in accordance with results from Dr. Estruch and colleagues that have shown that nonalcoholic beer presents a better anti-inflammatory effect comparing to other alcoholic beverages. In addition to this, we have observed an interesting correlation between some circulating and macrophage microRNAs that supports the notion that those circulating microRNAs could be released by the macrophages. Further studies are needed to clarify the effect of the alcohol.

3.6. Study limitations and future perspectives

The short sample size is the main limitation of this study, so it would be necessary to perform other interventions increasing the participants as well as including women to confirm the results obtained in this pilot study. In addition, the measurement of IX to check the beer consumption depend on the time where the participants drunk the beer and its suitability as biomarker of beer intake is limited. We allowed the participants to choose the moment and how they wanted to take the dose during the day leading to a great variability in IX levels.

4. PREDIMED-PLUS study

4.1 Analysis of the population after 1-year treatment

Our last objective was to analyze if a CR sustained along 1 year and based on a healthy dietary pattern (MD) plus PA modulated circulating and macrophages microRNAs. For that purpose, we selected 20 samples of each group of the PREDIMED-PLUS study. First, we observed that the intervention group had reduced anthropometrical parameters significantly. There was also a significant reduction in visceral fat, whereas the control group significantly increased visceral fat **(table 15).** This clearly suggest that the intervention is effective in the modification of obesity-related parameters. In biochemistry parameters, we did only observe a significant reduction in the levels of triglycerides **(table 15).**

Regarding lifestyle parameters, we observed that both groups increased the adherence to the MD. This is not surprising, given that participants in the control group are also encouraged to follow a MD. However, intervention group increased their PA level according to the specific recommendation that were given to each group.

4.2 Plasma microRNAs modifications after 1 year of treatment

SAM analysis of circulating microRNAs gave 8 microRNAs differentially expressed in control group and 6 in the intervention group, miR-200c, miR-301 and miR-30b were common for both groups. They were all upregulated after 1 year of intervention with a greater effect in the intervention group. However, a paired t-test did not confirm the significance. This intervention is not as acute as that in VOHF and miroBeer studies; instead is a chronic and multivariant intervention based on lifestyle instead of specific nutrients. In this sense, 1 year could be a too short time for a so multifactorial intervention to lead to significant changes in circulating microRNAs levels. The PREDIMED-PLUS intervention based on a complete lifestyle change involves the variation in more than one nutrient and variable, thus it introduces a higher variability that may require a larger sample size to have sensitivity enough to detect small changes in circulating microRNAs. On the other hand, after one year of intervention, participants are 1 year older, and we cannot rule out the possibility of an age-related modification of these microRNAs independently of the intervention (203).

Although changes were not significant, we observe and interesting trend to an increase in some plasma microRNAs. For instance, miR-30b, which is modulated by physical exercise and is related to cardiovascular risk in spinal cord-injured patients (370), miR-181 that belongs to a

family related to vascular inflammation and immunity (371) and miR-195 and miR-19a that are considered to be predictive of aging and respond to resistance exercise in men (303).

4.3. Macrophage microRNAs modifications after 1 year of treatment

SAM analyses showed 6 differentially modulated microRNAs in macrophages of both groups **(figure 38)**. However, after paired t-test none of the selected microRNAs in the control group remained significant.

On the other hand, paired t-test did show a significant downregulation of miR-130a and an almost significant downregulation of miR-30c in intervention group **(figure 39)**. In macrophages, Su S. *et al* observed that IL-4 and IL-13, interleukins with anti-inflammatory properties, lead to a downregulation of miR-130a (372) and, inflammatory factors as NF-KB increased its expression (373). Moreover, Sharma H *et al* showed that macrophages treatment with HIV and cocaine increased its expression (374). These results suggest that the intervention could be resulting in the release of anti-inflammatory interleukins that, in turn reduces miR-130a levels and that this intervention-mediated downregulation could result in an anti-inflammatory profile of macrophages. This hypothesis needs to be further investigated. On the other hand, miR-130a is a negative regulator of *TSC1* (TSC complex subunit 1), which is also a negative regulator of mTOR signaling (373). Therefore, miR-130a downregulation could be increasing TSC1-mediated inhibition of mTOR signaling, mirroring the effect of rapamycin, a supposed anti-aging drug (5).

Regarding to miR-30c, as it has been described before, it is an important regulator of VLDL (177) and its overexpression has an effect on the expression of genes related to lipid metabolism, increasing accumulation of triglycerides with lower levels of miR-30c (178). Moreover, reduction of miR-30c levels is associated to the production of pro-atherosclerotic signals like IL-1β release, caspase-3 expression and apoptosis that leads to inflammation and apoptosis in THP-1 cell line. (195). Additionally, Chen C *et al* showed that downregulation of miR-30c leads to the activation of *BECN1* (Beclin 1) that promotes autophagy and the pathogenesis of diabetic cardiomyopathy in mice and HEK293 cells treated with palmitate acid (375). Therefore, our results showing a downregulation of miR-30c by the treatment suggest that this reduction cold have a negative effect in lipid metabolism, CVD and atherosclerosis. This is inconsistent with a healthier macrophage microRNA profile after 1 year of treatment. Following studies are needed to clarify this controversy and elucidate the impact of the treatment-mediated reduction of miR-30c in macrophages.

The *in silico* functional analysis of the two macrophage microRNAs differentially expressed showed that they have 115 common target genes **(figure 40 and table 16).** Between them, the most relevant genes related to nutrient sensing pathways and CVD **(table 16)** are *AKAP10*, a gene involved in the activation of cAMP and for which polymorphisms have been found to be

associated with insulin resistance and T2DM (376) and *PRKAA2*, also involved in cAMP synthesis, which is 50% upregulated with an aerobic exercise intervention in dyslipemic mice (377). This is consistent with a potential modulation of this gene by our intervention that involves physical exercise. Other genes regulated by both microRNAs are LDLR and LPR6, involved in metabolism of LDL lipoproteins.

4.4 Senescence analysis

Due to the special characteristic of the recruited population, with a minimal age of 55 years old in men and 60 years old in women, we hypothesized that the aging of the population could be differential in the two groups and that such a different aging dynamic could be reflected in a differential progression of immune senescence. It is known that senescence is accompanied by a phenotypic change in the population of T cells with an increase in the ratio of senescent/non-senescent T cells. It has been described that senescent T cells loss gene and protein CD28 expression and become CD57 positive. Thus, the loss of *CD28*, coupled to an upregulation of CD57 has been used as biomarker of T cell senescence progression (378). We measured changes in the levels of both genes after 1 year of intervention in both, control and intervention groups **(figure 41).** We observed that intervention group presents higher levels of *CD28* and lower levels of CD57 comparing to control. These results could suggest that the proportion of senescent T cells in control individuals is higher than in individuals in the intervention group. Therefore, this suggest that the intervention could be delaying immune-senescence. Although specific studies are needed, we suggest that the intervention modifies microRNAs that, through the regulation of nutrient sensing pathways, delay the appearance of aging hallmarks, one of them is immune defeat. Different molecular studies to further analyze the direct impact of the modulation of these microRNAs in hallmarks of aging will be conducted in the near future.

4.5. Limitations and future perspectives

PREDIMED-PLUS is a clinical trial aimed to analyze the long-term effect of MD and physical activity on CVD in high cardiovascular risk patients with a follow-up of 8 years. The specific characteristics of this intervention with a non-acute intervention, make us to expect small changes in microRNA levels and the limited number of samples we have used to analyze microRNA levels have added high variability to results that difficult to observe significant small changes. For that reason, it would be necessary to include more samples to the analysis and to check the changes in microRNA profile in the next years to observe more sustained changes in microRNA profile. However, the observed modulation of miR-130a and miR-30c in macrophages is promising as it is the first report showing changes in microRNA levels in macrophages in a human intervention study based on a multifactorial intervention.

CONCLUSIONS / CONCLUSIONES

1. Substantial amounts of plant microRNAs have not been detected in EVOO and beer samples nor in in human plasma after the ingestion of an acute dose of EVOO. Therefore, our results do not support a cross-kingdom regulation mediated by plant exogenous microRNAs ingested with plant foods.

2. Different polyphenol-enriched EVOOs modulate circulating levels of microRNAs associated with CVD, insulin signaling, inflammation and aging. Among them, Let-7e, miR-17 and miR-20a (the two later belonging to the miR-17-92 cluster), microRNAs well described as modulators of nutrient sensing pathways and inflammation, are modified by the three types of functional oils.

3. A consistent dose-dependent response to polyphenol content was not observed in circulating microRNA levels, although microRNAs modulation mediated by L-EVOO and M-EVOO was more similar between them than with H-EVOO.

4. Circulating levels of miR-155, miR-328 and miR-92a are modulated differently by beer and non-alcoholic beer intake, suggesting an alcohol-specific effect, whereas miR-320 and miR-17 levels are downregulated by both types of beer, suggesting an alcohol-independent effect on these microRNAs. All of them could be good biomarkers of beer intake and could modulate IGF-1/PIK3/AKT/FOXO/mTOR signaling pathway and inflammation as shown by our *in silico* analysis.

5. Beer and non-alcoholic beer differently modulated the expression of miR-145, miR-17, miR-20a, miR-26b and miR-223 in macrophages, suggesting an alcohol specific effect. These results suggest that beer and non-alcoholic beer could modulate macrophage physiology through these microRNAs, involved in IGF-1/PIK3/AKT/FOXO/mTOR signaling pathway and inflammation as shown by our *in silico* analysis.

6. The effect of beer and non-alcoholic beer on the expression of circulating and macrophage microRNAs was different and suggests a pro-inflammatory microRNA profile and an anti-inflammatory microRNA profile of both beer types respectively.

7. Let-7e, miR-328, miR-26b and miR-92a circulating and macrophage levels were correlated suggesting that macrophages could be, in part, the source of these plasma microRNAs.

8. CR achieved with hypocaloric MD plus physical activity is more effective for weight loss, body composition balance, plasma triglycerides levels and, in general, cardiovascular health than only MD recommendations after 1 year of intervention.

9. CR achieved with hypocaloric MD plus physical activity modifies macrophage expression of miR-30c and miR-130a, involved in lipid metabolism and mTOR signaling regulation, suggesting that the beneficial effect of this intervention could be epigenetically mediated by the modulation of these microRNAs in macrophages. However, we did not find any significant modulation of circulating microRNAs, suggested that a more acute and long intervention is needed to detect substantial changes.

10. CR achieved with hypocaloric MD plus physical activity could delay age-related immune system defeat, due to the observed higher *CD28* and lower *CD57* levels in lymphocytes of intervention participants in comparison with control participants.

11. In general, let-7e and members of the miR-17-92 cluster (miR-17, mir-20a and miR-92a) were consistently modulated by different interventions both, in plasma and macrophages, suggesting that they are sensitively modulated by diet in humans and could be mediators of the effect of nutrients on cellular physiology in different tissues.

1. No se han detectado cantidades sustanciales de microRNAs de plantas en muestras de aceite de oliva virgen extra y cerveza ni en plasma humano después de la ingestión de una dosis aguda de aceite de oliva virgen extra. Por lo tanto, nuestros resultados no apoyan la hipótesis de una regulación cruzada entre diferentes reinos mediada por microRNAs exógenos de plantas ingeridas con alimentos vegetales.

2. Los diferentes aceites de oliva virgen extra enriquecidos con polifenoles modulan los niveles circulantes de microRNAs asociados con la enfermedad cardiovascular, la señalización de la insulina, la inflamación y el envejecimiento. Entre ellos, let-7e, miR-17 y miR-20a (los dos últimos pertenecientes al cluster miR-17-92), han sido descritos como moduladores de las vías sensoras de nutrientes y la inflamación y son modificados por los tres tipos de aceites funcionales.

3. No se observó una respuesta constante dosis-dependiente respecto al contenido de polifenoles en los niveles de microRNAs circulantes, aunque la modulación de los microRNAs mediada por L-EVOO y M-EVOO fue más similar entre ellos que con H-EVOO.

4. Los niveles circulantes de miR-155, miR-328 y miR-92a están modulados de manera diferente por el consumo de cerveza y cerveza no alcohólica, sugiriendo un efecto específico del alcohol, mientras que los niveles de miR-320 y miR-17 están reprimidos por ambos tipos de cerveza, sugiriendo un efecto independiente del alcohol en estos microRNAs. Todos ellos podrían ser buenos biomarcadores de la ingesta de cerveza y podrían modular la vía de señalización de IGF1/PIK3/AKT/FOXO/mTOR e inflamación como lo demuestra nuestro análisis *in silico*.

5. La cerveza y la cerveza sin alcohol modularon de manera diferente la expresión de miR-145, miR-17, miR-20a, miR-26b y miR-223 en macrófagos, sugiriendo un efecto específico del alcohol. Estos resultados sugieren que la cerveza y la cerveza sin alcohol podrían modular la fisiología de los macrófagos a través de estos microRNAs, que están involucrados en la vía de señalización e inflamación de IGF1/PIK3/AKT/FOXO/mTOR como lo muestra nuestro análisis *in silico*.

6. El efecto de la cerveza y la cerveza sin alcohol en la expresión de microRNAs circulantes y de macrófagos fue diferente y sugiere un perfil de microRNAs proinflamatorio en la cerveza con alcohol y un perfil de microRNAs antiinflamatorio en la cerveza sin alcohol.

7. Los niveles circulantes de let-7e, miR-328, miR-26b y miR-92a se correlacionaron con sus niveles de macrófagos, sugiriendo que los macrófagos podrían ser, en parte, la fuente de estos microRNAs en plasma.

8. La restricción calórica obtenida con la Dieta Mediterránea hipocalórica y la actividad física es más efectiva para la pérdida de peso, la composición corporal, la disminución de los niveles de triglicéridos en plasma y, en general, para la salud cardiovascular que las recomendaciones del Dieta Mediterránea después de 1 año de intervención.

9. La restricción calórica obtenida con la Dieta Mediterránea hipocalórica y la actividad física modifica la expresión de miR-30c y miR-130a en macrófagos, los cuáles están involucrados en el metabolismo de los lípidos y la regulación de la señalización de mTOR, sugiriendo un

efecto beneficioso. Sin embargo, no encontramos ninguna modulación significativa de microRNAs circulantes, lo que sugiere que se necesita una intervención más aguda y prolongada para detectar cambios sustanciales.

10. La restricción calórica obtenida con la Dieta Mediterránea hipocalórica y la actividad física podría retrasar la disfunción del sistema inmunológico relacionada con la edad, debido a los niveles más altos observados de *CD28* y *CD57* en los linfocitos de los participantes de intervención en comparación con los participantes control.

11. En general, let-7e y los miembros del cluster miR-17-92 (miR-17, mir-20a y miR-92a) fueron modulados consistentemente por diferentes intervenciones tanto en plasma como en macrófagos, sugiriendo que están modulados de manera importante por la dieta en humanos y podrían ser mediadores del efecto de los nutrientes sobre la fisiología celular en diferentes tejidos.

REFERENCES

1. Eurostat. **People in the EU: who are we and how do we live?** 2015.
2. INE. Nota de prensa. Proyecciones de Población 2016–2066. 2017.
3. Longo VD, Antebi A, Bartke A, Barzilai N, Brown-Borg HM, Caruso C, et al. Interventions to Slow Aging in Humans: Are We Ready? Aging cell. 2015;14(4):497-510.
4. Bektas A, Schurman SH, Sen R, Ferrucci L. Aging, inflammation and the environment. Exp Gerontol. 2017.
5. Novelle MG, Ali A, Dieguez C, Bernier M, de Cabo R. Metformin: A Hopeful Promise in Aging Research. Cold Spring Harbor perspectives in medicine. 2016;6(3):a025932.
6. Wu JW, Boudreau DM, Park Y, Simonds NI, Freedman AN. Commonly used diabetes and cardiovascular medications and cancer recurrence and cancer-specific mortality: a review of the literature. Expert opinion on drug safety. 2014;13(8):1071-99.
7. Ng TP, Feng L, Yap KB, Lee TS, Tan CH, Winblad B. Long-term metformin usage and cognitive function among older adults with diabetes. Journal of Alzheimer's disease : JAD. 2014;41(1):61-8.
8. Flynn JM, O'Leary MN, Zambataro CA, Academia EC, Presley MP, Garrett BJ, et al. Late-life rapamycin treatment reverses age-related heart dysfunction. Aging Cell. 2013;12(5):851-62.
9. Lattanzi G, Ortolani M, Columbaro M, Prencipe S, Mattioli E, Lanzarini C, et al. Lamins are rapamycin targets that impact human longevity: a study in centenarians. J Cell Sci. 2014;127(Pt 1):147-57.
10. Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, et al. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. Nature. 2009;460(7253):392-5.
11. Miller RA, Harrison DE, Astle CM, Fernandez E, Flurkey K, Han M, et al. Rapamycin-mediated lifespan increase in mice is dose and sex dependent and metabolically distinct from dietary restriction. Aging cell. 2014;13(3):468-77.
12. Jacobs JM, Cohen A, Ein-Mor E, Stessman J. Cholesterol, statins, and longevity from age 70 to 90 years. J Am Med Dir Assoc. 2013;14(12):883-8.
13. Strong R, Miller RA, Astle CM, Floyd RA, Flurkey K, Hensley KL, et al. Nordihydroguaiaretic acid and aspirin increase lifespan of genetically heterogeneous male mice. Aging Cell. 2008;7(5):641-50.
14. Hans H, Lone A, Aksenov V, Rollo CD. Impacts of metformin and aspirin on life history features and longevity of crickets: trade-offs versus cost-free life extension? Age (Dordr). 2015;37(2):31.
15. Ong AD, Uchino BN, Wethington E. Loneliness and Health in Older Adults: A Mini-Review and Synthesis. Gerontology. 2016;62(4):443-9.
16. Simioni C, Zauli G, Martelli AM, Vitale M, Sacchetti G, Gonelli A, et al. Oxidative stress: role of physical exercise and antioxidant nutraceuticals in adulthood and aging. Oncotarget. 2018;9(24):17181-98.
17. Vlietstra L, Hendrickx W, Waters DL. Exercise interventions in healthy older adults with sarcopenia: A systematic review and meta-analysis. Australas J Ageing. 2018.
18. Reuter I. Aging, physical activity, and disease prevention 2012. J Aging Res. 2012;2012:373294.
19. Ainslie PN, Cotter JD, George KP, Lucas S, Murrell C, Shave R, et al. Elevation in cerebral blood flow velocity with aerobic fitness throughout healthy human ageing. J Physiol. 2008;586(16):4005-10.
20. Yaffe K, Barnes D, Nevitt M, Lui LY, Covinsky K. A prospective study of physical activity and cognitive decline in elderly women: women who walk. Arch Intern Med. 2001;161(14):1703-8.
21. Denham J, Nelson CP, O'Brien BJ, Nankervis SA, Denniff M, Harvey JT, et al. Longer leukocyte telomeres are associated with ultra-endurance exercise independent of cardiovascular risk factors. PLoS One. 2013;8(7):e69377.
22. Balasubramanian P, Howell PR, Anderson RM. Aging and Caloric Restriction Research: A Biological Perspective With Translational Potential. EBioMedicine. 2017;21:37-44.
23. Fontana L, Partridge L. Promoting health and longevity through diet: from model organisms to humans. Cell. 2015;161(1):106-18.
24. Kaeberlein TL, Smith ED, Tsuchiya M, Welton KL, Thomas JH, Fields S, et al. Lifespan extension in Caenorhabditis elegans by complete removal of food. Aging cell. 2006;5(6):487-94.

25. Yang D, Lian T, Tu J, Gaur U, Mao X, Fan X, et al. LncRNA mediated regulation of aging pathways in Drosophila melanogaster during dietary restriction. Aging (Albany NY). 2016;8(9):2182-203.
26. Wierman MB, Maqani N, Strickler E, Li M, Smith JS. Caloric Restriction Extends Yeast Chronological Life Span by Optimizing the Snf1 (AMPK) Signaling Pathway. Mol Cell Biol. 2017;37(13).
27. Yu BP, Masoro EJ, Murata I, Bertrand HA, Lynd FT. Life span study of SPF Fischer 344 male rats fed ad libitum or restricted diets: longevity, growth, lean body mass and disease. Journal of gerontology. 1982;37(2):130-41.
28. Wohlgemuth SE, Seo AY, Marzetti E, Lees HA, Leeuwenburgh C. Skeletal muscle autophagy and apoptosis during aging: effects of calorie restriction and life-long exercise. Experimental gerontology. 2010;45(2):138-48.
29. Stockman MC, Thomas D, Burke J, Apovian CM. Intermittent Fasting: Is the Wait Worth the Weight? Curr Obes Rep. 2018;7(2):172-85.
30. Donati A, Recchia G, Cavallini G, Bergamini E. Effect of aging and anti-aging caloric restriction on the endocrine regulation of rat liver autophagy. The journals of gerontology Series A, Biological sciences and medical sciences. 2008;63(6):550-5.
31. Anson RM, Guo Z, de Cabo R, Iyun T, Rios M, Hagepanos A, et al. Intermittent fasting dissociates beneficial effects of dietary restriction on glucose metabolism and neuronal resistance to injury from calorie intake. Proceedings of the National Academy of Sciences of the United States of America. 2003;100(10):6216-20.
32. Varady KA, Roohk DJ, Loe YC, McEvoy-Hein BK, Hellerstein MK. Effects of modified alternate-day fasting regimens on adipocyte size, triglyceride metabolism, and plasma adiponectin levels in mice. Journal of lipid research. 2007;48(10):2212-9.
33. Swindell WR. Dietary restriction in rats and mice: a meta-analysis and review of the evidence for genotype-dependent effects on lifespan. Ageing research reviews. 2012;11(2):254-70.
34. Colman RJ, Anderson RM, Johnson SC, Kastman EK, Kosmatka KJ, Beasley TM, et al. Caloric restriction delays disease onset and mortality in rhesus monkeys. Science. 2009;325(5937):201-4.
35. Mattison JA, Roth GS, Beasley TM, Tilmont EM, Handy AM, Herbert RL, et al. Impact of caloric restriction on health and survival in rhesus monkeys from the NIA study. Nature. 2012;489(7415):318-21.
36. Mattison JA, Colman RJ, Beasley TM, Allison DB, Kemnitz JW, Roth GS, et al. Caloric restriction improves health and survival of rhesus monkeys. Nat Commun. 2017;8:14063.
37. Maxmen A. Calorie restriction falters in the long run. Nature. 2012;488(7413):569.
38. Soriano JB, Fernández Vázquez S, Carretero S, Puga González MD, Soriano C, Romaguera D, et al. Description of extreme longevity in the Balearic Islands: Exploring a potential Blue Zone in Menorca, Spain. Geriatr Gerontol Int. 2014;14(3):620-7.
39. Stefanadis CI. Unveiling the secrets of longevity: the Ikaria study. Hellenic J Cardiol. 2011;52(5):479-80.
40. Suzuki M, Wilcox BJ, Wilcox CD. Implications from and for food cultures for cardiovascular disease: longevity. Asia Pac J Clin Nutr. 2001;10(2):165-71.
41. Crous-Bou M, Fung TT, Prescott J, Julin B, Du M, Sun Q, et al. Mediterranean diet and telomere length in Nurses' Health Study: population based cohort study. BMJ. 2014;349:g6674.
42. Tuberoso CI, Boban M, Bifulco E, Budimir D, Pirisi FM. Antioxidant capacity and vasodilatory properties of Mediterranean food: the case of Cannonau wine, myrtle berries liqueur and strawberry-tree honey. Food Chem. 2013;140(4):686-91.
43. Heilbronn LK, de Jonge L, Frisard MI, DeLany JP, Larson-Meyer DE, Rood J, et al. Effect of 6-month calorie restriction on biomarkers of longevity, metabolic adaptation, and oxidative stress in overweight individuals: a randomized controlled trial. JAMA. 2006;295(13):1539-48.
44. Ravussin E, Redman LM, Rochon J, Das SK, Fontana L, Kraus WE, et al. A 2-Year Randomized Controlled Trial of Human Caloric Restriction: Feasibility and Effects on Predictors of Health Span and Longevity. J Gerontol A Biol Sci Med Sci. 2015;70(9):1097-104.

45. Larson-Meyer DE, Newcomer BR, Heilbronn LK, Volaufova J, Smith SR, Alfonso AJ, et al. Effect of 6-month calorie restriction and exercise on serum and liver lipids and markers of liver function. Obesity (Silver Spring). 2008;16(6):1355-62.
46. Lefevre M, Redman LM, Heilbronn LK, Smith JV, Martin CK, Rood JC, et al. Caloric restriction alone and with exercise improves CVD risk in healthy non-obese individuals. Atherosclerosis. 2009;203(1):206-13.
47. Tam CS, Frost EA, Xie W, Rood J, Ravussin E, Redman LM, et al. No effect of caloric restriction on salivary cortisol levels in overweight men and women. Metabolism. 2014;63(2):194-8.
48. Redman LM, Veldhuis JD, Rood J, Smith SR, Williamson D, Ravussin E, et al. The effect of caloric restriction interventions on growth hormone secretion in nonobese men and women. Aging Cell. 2010;9(1):32-9.
49. Redman LM, Heilbronn LK, Martin CK, de Jonge L, Williamson DA, Delany JP, et al. Metabolic and behavioral compensations in response to caloric restriction: implications for the maintenance of weight loss. PLoS One. 2009;4(2):e4377.
50. Williamson DA, Martin CK, Anton SD, York-Crowe E, Han H, Redman L, et al. Is caloric restriction associated with development of eating-disorder symptoms? Results from the CALERIE trial. Health Psychol. 2008;27(1S):S32-42.
51. Fontana L, Meyer TE, Klein S, Holloszy JO. Long-term calorie restriction is highly effective in reducing the risk for atherosclerosis in humans. Proc Natl Acad Sci U S A. 2004;101(17):6659-63.
52. Fontana L, Klein S, Holloszy JO. Effects of long-term calorie restriction and endurance exercise on glucose tolerance, insulin action, and adipokine production. Age (Dordr). 2010;32(1):97-108.
53. Soare A, Cangemi R, Omodei D, Holloszy JO, Fontana L. Long-term calorie restriction, but not endurance exercise, lowers core body temperature in humans. Aging (Albany NY). 2011;3(4):374-9.
54. Lettieri-Barbato D, Giovannetti E, Aquilano K. Effects of dietary restriction on adipose mass and biomarkers of healthy aging in human. Aging. 2016;8(12):3341-55.
55. Heilbronn LK, Smith SR, Martin CK, Anton SD, Ravussin E. Alternate-day fasting in nonobese subjects: effects on body weight, body composition, and energy metabolism. The American journal of clinical nutrition. 2005;81(1):69-73.
56. Heilbronn LK, Civitarese AE, Bogacka I, Smith SR, Hulver M, Ravussin E. Glucose tolerance and skeletal muscle gene expression in response to alternate day fasting. Obesity research. 2005;13(3):574-81.
57. Hoddy KK, Bhutani S, Phillips SA, Varady KA. Effects of different degrees of insulin resistance on endothelial function in obese adults undergoing alternate day fasting. Nutr Healthy Aging. 2016;4(1):63-71.
58. Harvie MN, Pegington M, Mattson MP, Frystyk J, Dillon B, Evans G, et al. The effects of intermittent or continuous energy restriction on weight loss and metabolic disease risk markers: a randomized trial in young overweight women. Int J Obes (Lond). 2011;35(5):714-27.
59. Harder-Lauridsen NM, Nielsen ST, Mann SP, Lyngbæk MP, Benatti FB, Langkilde AR, et al. The effect of alternate-day caloric restriction on the metabolic consequences of 8 days of bed rest in healthy lean men: a randomized trial. J Appl Physiol (1985). 2017;122(2):230-41.
60. Solon-Biet SM, McMahon AC, Ballard JW, Ruohonen K, Wu LE, Cogger VC, et al. The ratio of macronutrients, not caloric intake, dictates cardiometabolic health, aging, and longevity in ad libitum-fed mice. Cell metabolism. 2014;19(3):418-30.
61. Mirisola MG, Taormina G, Fabrizio P, Wei M, Hu J, Longo VD. Serine- and threonine/valine-dependent activation of PDK and Tor orthologs converge on Sch9 to promote aging. PLoS Genet. 2014;10(2):e1004113.
62. Mair W, Piper MD, Partridge L. Calories do not explain extension of life span by dietary restriction in Drosophila. PLoS Biol. 2005;3(7):e223.
63. Segall PE, Timiras PS. Patho-physiologic findings after chronic tryptophan deficiency in rats: a model for delayed growth and aging. Mech Ageing Dev. 1976;5(2):109-24.
64. Ooka H, Segall PE, Timiras PS. Histology and survival in age-delayed low-tryptophan-fed rats. Mech Ageing Dev. 1988;43(1):79-98.

65. Orentreich N, Matias JR, DeFelice A, Zimmerman JA. Low methionine ingestion by rats extends life span. J Nutr. 1993;123(2):269-74.
66. Miller RA, Buehner G, Chang Y, Harper JM, Sigler R, Smith-Wheelock M. Methionine-deficient diet extends mouse lifespan, slows immune and lens aging, alters glucose, T4, IGF-I and insulin levels, and increases hepatocyte MIF levels and stress resistance. Aging Cell. 2005;4(3):119-25.
67. Gallinetti J, Harputlugil E, Mitchell JR. Amino acid sensing in dietary-restriction-mediated longevity: roles of signal-transducing kinases GCN2 and TOR. Biochem J. 2013;449(1):1-10.
68. Fontana L, Adelaiye RM, Rastelli AL, Miles KM, Ciamporcero E, Longo VD, et al. Dietary protein restriction inhibits tumor growth in human xenograft models. Oncotarget. 2013;4(12):2451-61.
69. Fontana L, Weiss EP, Villareal DT, Klein S, Holloszy JO. Long-term effects of calorie or protein restriction on serum IGF-1 and IGFBP-3 concentration in humans. Aging cell. 2008;7(5):681-7.
70. Valls-Pedret C, Lamuela-Raventós RM, Medina-Remón A, Quintana M, Corella D, Pintó X, et al. Polyphenol-rich foods in the Mediterranean diet are associated with better cognitive function in elderly subjects at high cardiovascular risk. J Alzheimers Dis. 2012;29(4):773-82.
71. Menotti A, Keys A, Aravanis C, Blackburn H, Dontas A, Fidanza F, et al. Seven Countries Study. First 20-year mortality data in 12 cohorts of six countries. Ann Med. 1989;21(3):175-9.
72. Estruch R, Ros E, Salas-Salvadó J, Covas MI, Corella D, Arós F, et al. Retraction and Republication: Primary Prevention of Cardiovascular Disease with a Mediterranean Diet. N Engl J Med 2013;368:1279-90. N Engl J Med. 2018;378(25):2441-2.
73. Georgoulis M, Kontogianni MD, Yiannakouris N. Mediterranean diet and diabetes: prevention and treatment. Nutrients. 2014;6(4):1406-23.
74. Pallauf K, Giller K, Huebbe P, Rimbach G. Nutrition and healthy ageing: calorie restriction or polyphenol-rich "MediterrAsian" diet? Oxidative medicine and cellular longevity. 2013;2013:707421.
75. Covas MI, Nyyssonen K, Poulsen HE, Kaikkonen J, Zunft HJ, Kiesewetter H, et al. The effect of polyphenols in olive oil on heart disease risk factors: a randomized trial. Annals of internal medicine. 2006;145(5):333-41.
76. Samieri C, Féart C, Proust-Lima C, Peuchant E, Tzourio C, Stapf C, et al. Olive oil consumption, plasma oleic acid, and stroke incidence: the Three-City Study. Neurology. 2011;77(5):418-25.
77. Visioli F, Bernardini E. Extra virgin olive oil's polyphenols: biological activities. Current pharmaceutical design. 2011;17(8):786-804.
78. Garrido-Delgado R, Dobao-Prieto MeM, Arce L, Valcárcel M. Determination of volatile compounds by GC-IMS to assign the quality of virgin olive oil. Food Chem. 2015;187:572-9.
79. García-González Diego L, Aparicio-Ruiz R, Aparicio R. Virgin olive oil - Chemical implications on quality and health. European Journal of Lipid Science and Technology. 2008;110(7):602-7.
80. Gorzynik-Debicka M, Przychodzen P, Cappello F, Kuban-Jankowska A, Marino Gammazza A, Knap N, et al. Potential Health Benefits of Olive Oil and Plant Polyphenols. Int J Mol Sci. 2018;19(3).
81. Pedret A, Catalán Ú, Fernández-Castillejo S, Farràs M, Valls RM, Rubió L, et al. Impact of Virgin Olive Oil and Phenol-Enriched Virgin Olive Oils on the HDL Proteome in Hypercholesterolemic Subjects: A Double Blind, Randomized, Controlled, Cross-Over Clinical Trial (VOHF Study). PLoS One. 2015;10(6):e0129160.
82. Jemai H, El Feki A, Sayadi S. Antidiabetic and antioxidant effects of hydroxytyrosol and oleuropein from olive leaves in alloxan-diabetic rats. J Agric Food Chem. 2009;57(19):8798-804.
83. Granados-Principal S, Quiles JL, Ramirez-Tortosa CL, Sanchez-Rovira P, Ramirez-Tortosa MC. Hydroxytyrosol: from laboratory investigations to future clinical trials. Nutr Rev. 2010;68(4):191-206.
84. Fabiani R. Anti-cancer properties of olive oil secoiridoid phenols: a systematic review of in vivo studies. Food Funct. 2016;7(10):4145-59.
85. Dexter DT, Jenner P. Parkinson disease: from pathology to molecular disease mechanisms. Free Radic Biol Med. 2013;62:132-44.

86. Crespo MC, Tomé-Carneiro J, Pintado C, Dávalos A, Visioli F, Burgos-Ramos E. Hydroxytyrosol restores proper insulin signaling in an astrocytic model of Alzheimer's disease. Biofactors. 2017;43(4):540-8.
87. Cicerale S, Conlan XA, Sinclair AJ, Keast RS. Chemistry and health of olive oil phenolics. Crit Rev Food Sci Nutr. 2009;49(3):218-36.
88. Martín-Peláez S, Covas MI, Fitó M, Kušar A, Pravst I. Health effects of olive oil polyphenols: recent advances and possibilities for the use of health claims. Mol Nutr Food Res. 2013;57(5):760-71.
89. Di Francesco A, Falconi A, Di Germanio C, Micioni Di Bonaventura MV, Costa A, Caramuta S, et al. Extravirgin olive oil up-regulates CB1 tumor suppressor gene in human colon cancer cells and in rat colon via epigenetic mechanisms. J Nutr Biochem. 2015;26(3):250-8.
90. Konstantinidou V, Khymenets O, Fito M, De La Torre R, Anglada R, Dopazo A, et al. Characterization of human gene expression changes after olive oil ingestion: an exploratory approach. Folia Biol (Praha). 2009;55(3):85-91.
91. Konstantinidou V, Covas MI, Muñoz-Aguayo D, Khymenets O, de la Torre R, Saez G, et al. In vivo nutrigenomic effects of virgin olive oil polyphenols within the frame of the Mediterranean diet: a randomized controlled trial. FASEB J. 2010;24(7):2546-57.
92. Hittinger CT, Steele JL, Ryder DS. Diverse yeasts for diverse fermented beverages and foods. Curr Opin Biotechnol. 2018;49:199-206.
93. Bamforth CW. Progress in Brewing Science and Beer Production. Annu Rev Chem Biomol Eng. 2017;8:161-76.
94. Fernández-Solà J. Cardiovascular risks and benefits of moderate and heavy alcohol consumption. Nat Rev Cardiol. 2015;12(10):576-87.
95. Arranz S, Chiva-Blanch G, Valderas-Martínez P, Medina-Remón A, Lamuela-Raventós RM, Estruch R. Wine, beer, alcohol and polyphenols on cardiovascular disease and cancer. Nutrients. 2012;4(7):759-81.
96. Vilahur G, Casani L, Mendieta G, Lamuela-Raventos RM, Estruch R, Badimon L. Beer elicits vasculoprotective effects through Akt/eNOS activation. Eur J Clin Invest. 2014;44(12):1177-88.
97. Milligan SR, Kalita JC, Pocock V, Van De Kauter V, Stevens JF, Deinzer ML, et al. The endocrine activities of 8-prenylnaringenin and related hop (Humulus lupulus L.) flavonoids. J Clin Endocrinol Metab. 2000;85(12):4912-5.
98. Gerhauser C. Beer constituents as potential cancer chemopreventive agents. Eur J Cancer. 2005;41(13):1941-54.
99. Chung WG, Miranda CL, Stevens JF, Maier CS. Hop proanthocyanidins induce apoptosis, protein carbonylation, and cytoskeleton disorganization in human colorectal adenocarcinoma cells via reactive oxygen species. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association. 2009;47(4):827-36.
100. De Keukeleire D, De Cooman L, Rong H, Heyerick A, Kalita J, Milligan SR. Functional properties of hop polyphenols. Basic Life Sci. 1999;66:739-60.
101. Quifer-Rada P, Vallverdú-Queralt A, Martínez-Huélamo M, Chiva-Blanch G, Jáuregui O, Estruch R, et al. A comprehensive characterisation of beer polyphenols by high resolution mass spectrometry (LC-ESI-LTQ-Orbitrap-MS). Food Chem. 2015;169:336-43.
102. Costa R, Rodrigues I, Guardão L, Rocha-Rodrigues S, Silva C, Magalhães J, et al. Xanthohumol and 8-prenylnaringenin ameliorate diabetic-related metabolic dysfunctions in mice. J Nutr Biochem. 2017;45:39-47.
103. Stompor M, Uram Ł, Podgórski R. In Vitro Effect of 8-Prenylnaringenin and Naringenin on Fibroblasts and Glioblastoma Cells-Cellular Accumulation and Cytotoxicity. Molecules. 2017;22(7).
104. Mukai R, Horikawa H, Lin PY, Tsukumo N, Nikawa T, Kawamura T, et al. 8-Prenylnaringenin promotes recovery from immobilization-induced disuse muscle atrophy through activation of the Akt phosphorylation pathway in mice. Am J Physiol Regul Integr Comp Physiol. 2016;311(6):R1022-R31.
105. Vene R, Benelli R, Minghelli S, Astigiano S, Tosetti F, Ferrari N. Xanthohumol impairs human prostate cancer cell growth and invasion and diminishes the incidence and progression of advanced tumors in TRAMP mice. Molecular medicine. 2012;18:1292-302.

106. Hirata H, Yimin, Segawa S, Ozaki M, Kobayashi N, Shigyo T, et al. Xanthohumol prevents atherosclerosis by reducing arterial cholesterol content via CETP and apolipoprotein E in CETP-transgenic mice. PLoS One. 2012;7(11):e49415.
107. Doddapattar P, Radovic B, Patankar JV, Obrowsky S, Jandl K, Nusshold C, et al. Xanthohumol ameliorates atherosclerotic plaque formation, hypercholesterolemia, and hepatic steatosis in ApoE-deficient mice. Mol Nutr Food Res. 2013;57(10):1718-28.
108. Kirkwood JS, Legette LL, Miranda CL, Jiang Y, Stevens JF. A metabolomics-driven elucidation of the anti-obesity mechanisms of xanthohumol. J Biol Chem. 2013;288(26):19000-13.
109. Valls-Belles V, Torres C, Muniz P, Codoner-Franch P. Effect of beer consumption on levels of complex I and complex IV liver and heart mitochondrial enzymes and coenzymes Q9 and Q10 in adriamycin-treated rats. European journal of nutrition. 2010;49(3):181-7.
110. Vilahur G, Casani L, Guerra JM, Badimon L. Intake of fermented beverages protect against acute myocardial injury: target organ cardiac effects and vasculoprotective effects. Basic Res Cardiol. 2012;107(5):291.
111. Lopez-Otin C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. Cell. 2013;153(6):1194-217.
112. Efeyan A, Comb WC, Sabatini DM. Nutrient-sensing mechanisms and pathways. Nature. 2015;517(7534):302-10.
113. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. Cell. 2012;149(2):274-93.
114. Carroll B, Hewitt G, Korolchuk VI. Autophagy and ageing: implications for age-related neurodegenerative diseases. Essays Biochem. 2013;55:119-31.
115. Altintas O, Park S, Lee SJ. The role of insulin/IGF-1 signaling in the longevity of model invertebrates, C. elegans and D. melanogaster. BMB reports. 2016;49(2):81-92.
116. Sharples AP, Hughes DC, Deane CS, Saini A, Selman C, Stewart CE. Longevity and skeletal muscle mass: the role of IGF signalling, the sirtuins, dietary restriction and protein intake. Aging Cell. 2015;14(4):511-23.
117. Johnson SC, Rabinovitch PS, Kaeberlein M. mTOR is a key modulator of ageing and age-related disease. Nature. 2013;493(7432):338-45.
118. Wang R, Dillon CP, Shi LZ, Milasta S, Carter R, Finkelstein D, et al. The transcription factor Myc controls metabolic reprogramming upon T lymphocyte activation. Immunity. 2011;35(6):871-82.
119. Carroll B, Dunlop EA. The lysosome: a crucial hub for AMPK and mTORC1 signalling. Biochem J. 2017;474(9):1453-66.
120. Nagy G, Kovacs-Nagy R, Kereszturi E, Somogyi A, Szekely A, Nemeth N, et al. Association of hypoxia inducible factor-1 alpha gene polymorphism with both type 1 and type 2 diabetes in a Caucasian (Hungarian) sample. BMC Med Genet. 2009;10:79.
121. Laybutt DR, Weir GC, Kaneto H, Lebet J, Palmiter RD, Sharma A, et al. Overexpression of c-Myc in beta-cells of transgenic mice causes proliferation and apoptosis, downregulation of insulin gene expression, and diabetes. Diabetes. 2002;51(6):1793-804.
122. Tia N, Singh AK, Pandey P, Azad CS, Chaudhary P, Gambhir IS. Role of Forkhead Box O (FOXO) transcription factor in aging and diseases. Gene. 2018;648:97-105.
123. Schug TT, Li X. Sirtuin 1 in lipid metabolism and obesity. Ann Med. 2011;43(3):198-211.
124. Davalos A, Goedeke L, Smibert P, Ramirez CM, Warrier NP, Andreo U, et al. miR-33a/b contribute to the regulation of fatty acid metabolism and insulin signaling. Proceedings of the National Academy of Sciences of the United States of America. 2011;108(22):9232-7.
125. Canto C, Auwerx J. Targeting sirtuin 1 to improve metabolism: all you need is NAD(+)? Pharmacological reviews. 2012;64(1):166-87.
126. Tullet JM, Hertweck M, An JH, Baker J, Hwang JY, Liu S, et al. Direct inhibition of the longevity-promoting factor SKN-1 by insulin-like signaling in C. elegans. Cell. 2008;132(6):1025-38.
127. Uno M, Honjoh S, Matsuda M, Hoshikawa H, Kishimoto S, Yamamoto T, et al. A fasting-responsive signaling pathway that extends life span in C. elegans. Cell reports. 2013;3(1):79-91.

128. Giannakou ME, Goss M, Partridge L. Role of dFOXO in lifespan extension by dietary restriction in Drosophila melanogaster: not required, but its activity modulates the response. Aging Cell. 2008;7(2):187-98.
129. Warr MR, Binnewies M, Flach J, Reynaud D, Garg T, Malhotra R, et al. FOXO3A directs a protective autophagy program in haematopoietic stem cells. Nature. 2013;494(7437):323-7.
130. Shimokawa I, Komatsu T, Hayashi N, Kim SE, Kawata T, Park S, et al. The life-extending effect of dietary restriction requires Foxo3 in mice. Aging Cell. 2015;14(4):707-9.
131. Bao JM, Song XL, Hong YQ, Zhu HL, Li C, Zhang T, et al. Association between FOXO3A gene polymorphisms and human longevity: a meta-analysis. Asian J Androl. 2014;16(3):446-52.
132. Willcox BJ, Tranah GJ, Chen R, Morris BJ, Masaki KH, He Q, et al. The FoxO3 gene and cause-specific mortality. Aging Cell. 2016;15(4):617-24.
133. Mercken EM, Crosby SD, Lamming DW, JeBailey L, Krzysik-Walker S, Villareal DT, et al. Calorie restriction in humans inhibits the PI3K/AKT pathway and induces a younger transcription profile. Aging cell. 2013;12(4):645-51.
134. Kapahi P, Zid BM, Harper T, Koslover D, Sapin V, Benzer S. Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway. Curr Biol. 2004;14(10):885-90.
135. Jia K, Chen D, Riddle DL. The TOR pathway interacts with the insulin signaling pathway to regulate C. elegans larval development, metabolism and life span. Development. 2004;131(16):3897-906.
136. Honjoh S, Yamamoto T, Uno M, Nishida E. Signalling through RHEB-1 mediates intermittent fasting-induced longevity in C. elegans. Nature. 2009;457(7230):726-30.
137. Lee GD, Wilson MA, Zhu M, Wolkow CA, de Cabo R, Ingram DK, et al. Dietary deprivation extends lifespan in Caenorhabditis elegans. Aging cell. 2006;5(6):515-24.
138. Suh Y, Atzmon G, Cho MO, Hwang D, Liu B, Leahy DJ, et al. Functionally significant insulin-like growth factor I receptor mutations in centenarians. Proceedings of the National Academy of Sciences of the United States of America. 2008;105(9):3438-42.
139. Milman S, Atzmon G, Huffman DM, Wan J, Crandall JP, Cohen P, et al. Low insulin-like growth factor-1 level predicts survival in humans with exceptional longevity. Aging cell. 2014;13(4):769-71.
140. Allard JS, Heilbronn LK, Smith C, Hunt ND, Ingram DK, Ravussin E, et al. In vitro cellular adaptations of indicators of longevity in response to treatment with serum collected from humans on calorie restricted diets. PLoS One. 2008;3(9):e3211.
141. Lin SJ, Defossez PA, Guarente L. Requirement of NAD and SIR2 for life-span extension by calorie restriction in Saccharomyces cerevisiae. Science. 2000;289(5487):2126-8.
142. Scheibye-Knudsen M, Mitchell SJ, Fang EF, Iyama T, Ward T, Wang J, et al. A high-fat diet and NAD(+) activate Sirt1 to rescue premature aging in cockayne syndrome. Cell Metab. 2014;20(5):840-55.
143. Satoh A, Brace CS, Ben-Josef G, West T, Wozniak DF, Holtzman DM, et al. SIRT1 promotes the central adaptive response to diet restriction through activation of the dorsomedial and lateral nuclei of the hypothalamus. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2010;30(30):10220-32.
144. Satoh A, Brace CS, Rensing N, Cliften P, Wozniak DF, Herzog ED, et al. Sirt1 extends life span and delays aging in mice through the regulation of Nk2 homeobox 1 in the DMH and LH. Cell metabolism. 2013;18(3):416-30.
145. Mouchiroud L, Houtkooper RH, Auwerx J. NAD^+ metabolism: a therapeutic target for age-related metabolic disease. Crit Rev Biochem Mol Biol. 2013;48(4):397-408.
146. Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, et al. Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. Nature. 2003;425(6954):191-6.
147. Roush S, Slack FJ. The let-7 family of microRNAs. Trends Cell Biol. 2008;18(10):505-16.
148. Winter J, Jung S, Keller S, Gregory RI, Diederichs S. Many roads to maturity: microRNA biogenesis pathways and their regulation. Nature cell biology. 2009;11(3):228-34.
149. Ambros V. The functions of animal microRNAs. Nature. 2004;431(7006):350-5.
150. Carthew RW, Sontheimer EJ. Origins and Mechanisms of miRNAs and siRNAs. Cell. 2009;136(4):642-55.

151. Lee Y, Jeon K, Lee JT, Kim S, Kim VN. MicroRNA maturation: stepwise processing and subcellular localization. EMBO J. 2002;21(17):4663-70.
152. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, et al. The nuclear RNase III Drosha initiates microRNA processing. Nature. 2003;425(6956):415-9.
153. Han J, Lee Y, Yeom KH, Nam JW, Heo I, Rhee JK, et al. Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. Cell. 2006;125(5):887-901.
154. Bohnsack MT, Czaplinski K, Gorlich D. Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. RNA. 2004;10(2):185-91.
155. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009;136(2):215-33.
156. Zhang H, Kolb FA, Jaskiewicz L, Westhof E, Filipowicz W. Single processing center models for human Dicer and bacterial RNase III. Cell. 2004;118(1):57-68.
157. Havens MA, Reich AA, Duelli DM, Hastings ML. Biogenesis of mammalian microRNAs by a non-canonical processing pathway. Nucleic Acids Res. 2012;40(10):4626-40.
158. Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet. 2008;9(2):102-14.
159. Guo ZW, Xie C, Yang JR, Li JH, Yang JH, Zheng L. MtiBase: a database for decoding microRNA target sites located within CDS and 5'UTR regions from CLIP-Seq and expression profile datasets. Database (Oxford). 2015;2015.
160. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res. 2009;19(1):92-105.
161. Maegdefessel L. The emerging role of microRNAs in cardiovascular disease. Journal of internal medicine. 2014;276(6):633-44.
162. Aryal B, Singh AK, Rotllan N, Price N, Fernández-Hernando C. MicroRNAs and lipid metabolism. Curr Opin Lipidol. 2017;28(3):273-80.
163. Ou Z, Wada T, Gramignoli R, Li S, Strom SC, Huang M, et al. MicroRNA hsa-miR-613 targets the human LXRα gene and mediates a feedback loop of LXRα autoregulation. Mol Endocrinol. 2011;25(4):584-96.
164. Lynn FC. Meta-regulation: microRNA regulation of glucose and lipid metabolism. Trends Endocrinol Metab. 2009;20(9):452-9.
165. Rayner KJ, Suarez Y, Davalos A, Parathath S, Fitzgerald ML, Tamehiro N, et al. MiR-33 contributes to the regulation of cholesterol homeostasis. Science. 2010;328(5985):1570-3.
166. Saeidimehr S, Ebrahimi A, Saki N, Goodarzi P, Rahim F. MicroRNA-Based Linkage between Aging and Cancer: from Epigenetics View Point. Cell J. 2016;18(2):117-26.
167. Frost RJ, Olson EN. Control of glucose homeostasis and insulin sensitivity by the Let-7 family of microRNAs. Proc Natl Acad Sci U S A. 2011;108(52):21075-80.
168. Zhu H, Shyh-Chang N, Segrè AV, Shinoda G, Shah SP, Einhorn WS, et al. The Lin28/let-7 axis regulates glucose metabolism. Cell. 2011;147(1):81-94.
169. Wilfred BR, Wang WX, Nelson PT. Energizing miRNA research: a review of the role of miRNAs in lipid metabolism, with a prediction that miR-103/107 regulates human metabolic pathways. Mol Genet Metab. 2007;91(3):209-17.
170. Zhu H, Leung SW. Identification of microRNA biomarkers in type 2 diabetes: a meta-analysis of controlled profiling studies. Diabetologia. 2015;58(5):900-11.
171. Horie T, Ono K, Horiguchi M, Nishi H, Nakamura T, Nagao K, et al. MicroRNA-33 encoded by an intron of sterol regulatory element-binding protein 2 (Srebp2) regulates HDL in vivo. Proc Natl Acad Sci U S A. 2010;107(40):17321-6.
172. Goedeke L, Rotllan N, Canfrán-Duque A, Aranda JF, Ramírez CM, Araldi E, et al. MicroRNA-148a regulates LDL receptor and ABCA1 expression to control circulating lipoprotein levels. Nat Med. 2015;21(11):1280-9.
173. Elmen J, Lindow M, Schutz S, Lawrence M, Petri A, Obad S, et al. LNA-mediated microRNA silencing in non-human primates. Nature. 2008;452(7189):896-9.
174. Esau C, Davis S, Murray SF, Yu XX, Pandey SK, Pear M, et al. miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. Cell metabolism. 2006;3(2):87-98.

175. Chartoumpekis DV, Zaravinos A, Ziros PG, Iskrenova RP, Psyrogiannis AI, Kyriazopoulou VE, et al. Differential Expression of MicroRNAs in Adipose Tissue after Long-Term High-Fat Diet-Induced Obesity in Mice. PloS one. 2012;7(4):e34872.
176. Tessitore A, Cicciarelli G, Del Vecchio F, Gaggiano A, Verzella D, Fischietti M, et al. MicroRNA expression analysis in high fat diet-induced NAFLD-NASH-HCC progression: study on C57BL/6J mice. BMC Cancer. 2016;16:3.
177. Soh J, Iqbal J, Queiroz J, Fernandez-Hernando C, Hussain MM. MicroRNA-30c reduces hyperlipidemia and atherosclerosis in mice by decreasing lipid synthesis and lipoprotein secretion. Nature medicine. 2013;19(7):892-900.
178. Gil-Zamorano J, Martin R, Daimiel L, Richardson K, Giordano E, Nicod N, et al. Docosahexaenoic acid modulates the enterocyte Caco-2 cell expression of microRNAs involved in lipid metabolism. J Nutr. 2014;144(5):575-85.
179. Daimiel-Ruiz L, Klett-Mingo M, Konstantinidou V, Mico V, Aranda JF, Garcia B, et al. Dietary lipids modulate the expression of miR-107, a miRNA that regulates the circadian system. Molecular nutrition & food research. 2015;59(9):1865-78.
180. Sangiao-Alvarellos S, Pena-Bello L, Manfredi-Lozano M, Tena-Sempere M, Cordido F. Perturbation of hypothalamic microRNA expression patterns in male rats after metabolic distress: impact of obesity and conditions of negative energy balance. Endocrinology. 2014;155(5):1838-50.
181. Vickers KC, Landstreet SR, Levin MG, Shoucri BM, Toth CL, Taylor RC, et al. MicroRNA-223 coordinates cholesterol homeostasis. Proc Natl Acad Sci U S A. 2014;111(40):14518-23.
182. Canfran-Duque A, Rotllan N, Zhang X, Fernandez-Fuertes M, Ramirez-Hidalgo C, Araldi E, et al. Macrophage deficiency of miR-21 promotes apoptosis, plaque necrosis, and vascular inflammation during atherogenesis. EMBO molecular medicine. 2017;9(9):1244-62.
183. Ortega FJ, Cardona-Alvarado MI, Mercader JM, Moreno-Navarrete JM, Moreno M, Sabater M, et al. Circulating profiling reveals the effect of a polyunsaturated fatty acid-enriched diet on common microRNAs. J Nutr Biochem. 2015;26(10):1095-101.
184. Tabet F, Cuesta Torres LF, Ong KL, Shrestha S, Choteau SA, Barter PJ, et al. High-Density Lipoprotein-Associated miR-223 Is Altered after Diet-Induced Weight Loss in Overweight and Obese Males. PLoS One. 2016;11(3):e0151061.
185. Ma X, Becker Buscaglia LE, Barker JR, Li Y. MicroRNAs in NF-kappaB signaling. J Mol Cell Biol. 2011;3(3):159-66.
186. Zampetaki A, Willeit P, Drozdov I, Kiechl S, Mayr M. Profiling of circulating microRNAs: from single biomarkers to re-wired networks. Cardiovasc Res. 2012;93(4):555-62.
187. Arrese M, Eguchi A, Feldstein AE. Circulating microRNAs: emerging biomarkers of liver disease. Seminars in liver disease. 2015;35(1):43-54.
188. Liu G, Niu X, Meng X, Zhang Z. Sensitive miRNA markers for the detection and management of NSTEMI acute myocardial infarction patients. J Thorac Dis. 2018;10(6):3206-15.
189. Li YQ, Zhang MF, Wen HY, Hu CL, Liu R, Wei HY, et al. Comparing the diagnostic values of circulating microRNAs and cardiac troponin T in patients with acute myocardial infarction. Clinics (Sao Paulo). 2013;68(1):75-80.
190. Goren Y, Kushnir M, Zafrir B, Tabak S, Lewis BS, Amir O. Serum levels of microRNAs in patients with heart failure. European journal of heart failure. 2012;14(2):147-54.
191. Tijsen AJ, Creemers EE, Moerland PD, de Windt LJ, van der Wal AC, Kok WE, et al. MiR423-5p as a circulating biomarker for heart failure. Circulation research. 2010;106(6):1035-9.
192. Kontaraki JE, Marketou ME, Zacharis EA, Parthenakis FI, Vardas PE. MicroRNA-9 and microRNA-126 expression levels in patients with essential hypertension: potential markers of target-organ damage. J Am Soc Hypertens. 2014;8(6):368-75.
193. Yilmaz SG, Isbir S, Kunt AT, Isbir T. Circulating microRNAs as Novel Biomarkers for Atherosclerosis. In Vivo. 2018;32(3):561-5.
194. Caserta S, Mengozzi M, Kern F, Newbury SF, Ghezzi P, Llewelyn MJ. Severity of Systemic Inflammatory Response Syndrome Affects the Blood Levels of Circulating Inflammatory-Relevant MicroRNAs. Front Immunol. 2017;8:1977.

195. Ceolotto G, Giannella A, Albiero M, Kuppusamy M, Radu C, Simioni P, et al. miR-30c-5p regulates macrophage-mediated inflammation and pro-atherosclerosis pathways. Cardiovasc Res. 2017;113(13):1627-38.
196. Bellissimo T, Russo E, Ganci F, Vico C, Sacconi A, Longo F, et al. Circulating miR-21-5p and miR-148a-3p as emerging non-invasive biomarkers in thymic epithelial tumors. Cancer Biol Ther. 2016;17(1):79-82.
197. Zhang H, Mao F, Shen T, Luo Q, Ding Z, Qian L, et al. Plasma miR-145, miR-20a, miR-21 and miR-223 as novel biomarkers for screening early-stage non-small cell lung cancer. Oncol Lett. 2017;13(2):669-76.
198. Li Y, Yao M, Zhou Q, Cheng Y, Che L, Xu J, et al. Dynamic Regulation of Circulating microRNAs During Acute Exercise and Long-Term Exercise Training in Basketball Athletes. Front Physiol. 2018;9:282.
199. de Gonzalo-Calvo D, Dávalos A, Montero A, García-González Á, Tyshkovska I, González-Medina A, et al. Circulating inflammatory miRNA signature in response to different doses of aerobic exercise. J Appl Physiol (1985). 2015;119(2):124-34.
200. Cosar E, Mamillapalli R, Moridi I, Duleba A, Taylor HS. Serum MicroRNA Biomarkers Regulated by Simvastatin in a Primate Model of Endometriosis. Reprod Sci. 2018:1933719118765971.
201. Dickinson BA, Semus HM, Montgomery RL, Stack C, Latimer PA, Lewton SM, et al. Plasma microRNAs serve as biomarkers of therapeutic efficacy and disease progression in hypertension-induced heart failure. Eur J Heart Fail. 2013;15(6):650-9.
202. Suzuki HI, Yamagata K, Sugimoto K, Iwamoto T, Kato S, Miyazono K. Modulation of microRNA processing by p53. Nature. 2009;460(7254):529-33.
203. Serna E, Gambini J, Borras C, Abdelaziz KM, Mohammed K, Belenguer A, et al. Centenarians, but not octogenarians, up-regulate the expression of microRNAs. Sci Rep. 2012;2:961.
204. Ugalde AP, Espanol Y, Lopez-Otin C. Micromanaging aging with miRNAs: new messages from the nuclear envelope. Nucleus. 2011;2(6):549-55.
205. Olivieri F, Capri M, Bonafè M, Morsiani C, Jung HJ, Spazzafumo L, et al. Circulating miRNAs and miRNA shuttles as biomarkers: Perspective trajectories of healthy and unhealthy aging. Mech Ageing Dev. 2016.
206. Pang J, Xiong H, Yang H, Ou Y, Xu Y, Huang Q, et al. Circulating miR-34a levels correlate with age-related hearing loss in mice and humans. Experimental gerontology. 2016;76:58-67.
207. Tugay K, Guay C, Marques AC, Allagnat F, Locke JM, Harries LW, et al. Role of microRNAs in the age-associated decline of pancreatic beta cell function in rat islets. Diabetologia. 2016;59(1):161-9.
208. Berber P, Grassmann F, Kiel C, Weber BH. An Eye on Age-Related Macular Degeneration: The Role of MicroRNAs in Disease Pathology. Molecular diagnosis & therapy. 2017;21(1):31-43.
209. Gong X, Wang H, Ye Y, Shu Y, Deng Y, He X, et al. miR-124 regulates cell apoptosis and autophagy in dopaminergic neurons and protects them by regulating AMPK/mTOR pathway in Parkinson's disease. Am J Transl Res. 2016;8(5):2127-37.
210. Harada M, Jinnin M, Wang Z, Hirano A, Tomizawa Y, Kira T, et al. The expression of miR-124 increases in aged skin to cause cell senescence and it decreases in squamous cell carcinoma. Bioscience trends. 2017;10(6):454-9.
211. Teteloshvili N, Kluiver J, van der Geest KS, van der Lei RJ, Jellema P, Pawelec G, et al. Age-Associated Differences in MiRNA Signatures Are Restricted to CD45RO Negative T Cells and Are Associated with Changes in the Cellular Composition, Activation and Cellular Ageing. PloS one. 2015;10(9):e0137556.
212. Xu F, Pang L, Cai X, Liu X, Yuan S, Fan X, et al. let-7-repressesed Shc translation delays replicative senescence. Aging Cell. 2014;13(1):185-92.
213. Hyun S, Lee JH, Jin H, Nam J, Namkoong B, Lee G, et al. Conserved MicroRNA miR-8/miR-200 and its target USH/FOG2 control growth by regulating PI3K. Cell. 2009;139(6):1096-108.
214. Ma M, Wang X, Chen X, Cai R, Chen F, Dong W, et al. MicroRNA-432 targeting E2F3 and P55PIK inhibits myogenesis through PI3K/AKT/mTOR signaling pathway. RNA Biol. 2017:0.

215. Han C, Shen JK, Hornicek FJ, Kan Q, Duan Z. Regulation of microRNA-1 (miR-1) expression in human cancer. Biochim Biophys Acta. 2017;1860(2):227-32.
216. Mariño G, Ugalde AP, Fernández AF, Osorio FG, Fueyo A, Freije JM, et al. Insulin-like growth factor 1 treatment extends longevity in a mouse model of human premature aging by restoring somatotroph axis function. Proc Natl Acad Sci U S A. 2010;107(37):16268-73.
217. Gao H, Deng H, Xu H, Yang Q, Zhou Y, Zhang J, et al. MicroRNA-223 promotes mast cell apoptosis by targeting the insulin-like growth factor 1 receptor. Exp Ther Med. 2016;11(6):2171-6.
218. Xie R, Lin X, Du T, Xu K, Shen H, Wei F, et al. Targeted Disruption of miR-17-92 Impairs Mouse Spermatogenesis by Activating mTOR Signaling Pathway. Medicine (Baltimore). 2016;95(7):e2713.
219. Grillari J, Hackl M, Grillari-Voglauer R. miR-17-92 cluster: ups and downs in cancer and aging. Biogerontology. 2010;11(4):501-6.
220. Law PT, Ching AK, Chan AW, Wong QW, Wong CK, To KF, et al. MiR-145 modulates multiple components of the insulin-like growth factor pathway in hepatocellular carcinoma. Carcinogenesis. 2012;33(6):1134-41.
221. Budzinska M, Owczarz M, Pawlik-Pachucka E, Roszkowska-Gancarz M, Slusarczyk P, Puzianowska-Kuznicka M. miR-96, miR-145 and miR-9 expression increases, and IGF-1R and FOXO1 expression decreases in peripheral blood mononuclear cells of aging humans. BMC Geriatr. 2016;16(1):200.
222. Meyer SU, Thirion C, Polesskaya A, Bauersachs S, Kaiser S, Krause S, et al. TNF-α and IGF1 modify the microRNA signature in skeletal muscle cell differentiation. Cell Commun Signal. 2015;13:4.
223. Olivieri F, Ahtiainen M, Lazzarini R, Pöllänen E, Capri M, Lorenzi M, et al. Hormone replacement therapy enhances IGF-1 signaling in skeletal muscle by diminishing miR-182 and miR-223 expressions: a study on postmenopausal monozygotic twin pairs. Aging Cell. 2014;13(5):850-61.
224. Liu ZQ, Fu WQ, Zhao S, Zhao X. Regulation of insulin-like growth factor 1 receptor signaling by microRNA-4458 in the development of lumbar disc degeneration. Am J Transl Res. 2016;8(5):2309-16.
225. Li X, Sun X, Wu J, Li Z. MicroRNA-613 suppresses proliferation, migration and invasion of osteosarcoma by targeting c-MET. Am J Cancer Res. 2016;6(12):2869-79.
226. Liu P, Hu Y, Ma L, Du M, Xia L, Hu Z. miR-425 inhibits melanoma metastasis through repression of PI3K-Akt pathway by targeting IGF-1. Biomed Pharmacother. 2015;75:51-7.
227. Ihle MA, Trautmann M, Kuenstlinger H, Huss S, Heydt C, Fassunke J, et al. miRNA-221 and miRNA-222 induce apoptosis via the KIT/AKT signalling pathway in gastrointestinal stromal tumours. Mol Oncol. 2015;9(7):1421-33.
228. Iekushi K, Seeger F, Assmus B, Zeiher AM, Dimmeler S. Regulation of cardiac microRNAs by bone marrow mononuclear cell therapy in myocardial infarction. Circulation. 2012;125(14):1765-73, S1-7.
229. Chen L, Wang Q, Wang GD, Wang HS, Huang Y, Liu XM, et al. miR-16 inhibits cell proliferation by targeting IGF1R and the Raf1-MEK1/2-ERK1/2 pathway in osteosarcoma. FEBS Lett. 2013;587(9):1366-72.
230. Xie H, Lei N, Gong AY, Chen XM, Hu G. Cryptosporidium parvum induces SIRT1 expression in host epithelial cells through downregulating let-7i. Hum Immunol. 2014;75(8):760-5.
231. Menghini R, Casagrande V, Cardellini M, Martelli E, Terrinoni A, Amati F, et al. MicroRNA 217 modulates endothelial cell senescence via silent information regulator 1. Circulation. 2009;120(15):1524-32.
232. Kurylowicz A, Owczarz M, Polosak J, Jonas MI, Lisik W, Jonas M, et al. SIRT1 and SIRT7 expression in adipose tissues of obese and normal-weight individuals is regulated by microRNAs but not by methylation status. Int J Obes (Lond). 2016;40(11):1635-42.
233. Yamac AH, Kucukbuzcu S, Ozansoy M, Gok O, Oz K, Erturk M, et al. Altered expression of micro-RNA 199a and increased levels of cardiac SIRT1 protein are associated with the occurrence of atrial fibrillation after coronary artery bypass graft surgery. Cardiovasc Pathol. 2016;25(3):232-6.
234. Yu JM, Wu X, Gimble JM, Guan X, Freitas MA, Bunnell BA. Age-related changes in mesenchymal stem cells derived from rhesus macaque bone marrow. Aging Cell. 2011;10(1):66-79.

235. Xue Y, Wei Z, Ding H, Wang Q, Zhou Z, Zheng S, et al. MicroRNA-19b/221/222 induces endothelial cell dysfunction via suppression of PGC-1α in the progression of atherosclerosis. Atherosclerosis. 2015;241(2):671-81.
236. Zhang H, Guan M, Townsend KL, Huang TL, An D, Yan X, et al. MicroRNA-455 regulates brown adipogenesis via a novel HIF1an-AMPK-PGC1α signaling network. EMBO Rep. 2015;16(10):1378-93.
237. Nidadavolu LS, Niedernhofer LJ, Khan SA. Identification of microRNAs dysregulated in cellular senescence driven by endogenous genotoxic stress. Aging (Albany NY). 2013;5(6):460-73.
238. Chen MB, Wei MX, Han JY, Wu XY, Li C, Wang J, et al. MicroRNA-451 regulates AMPK/mTORC1 signaling and fascin1 expression in HT-29 colorectal cancer. Cell Signal. 2014;26(1):102-9.
239. Li G, Yu M, Lee WW, Tsang M, Krishnan E, Weyand CM, et al. Decline in miR-181a expression with age impairs T cell receptor sensitivity by increasing DUSP6 activity. Nat Med. 2012;18(10):1518-24.
240. Kawashima I, Mitsumori T, Nozaki Y, Yamamoto T, Shobu-Sueki Y, Nakajima K, et al. Negative regulation of the LKB1/AMPK pathway by ERK in human acute myeloid leukemia cells. Exp Hematol. 2015;43(7):524-33.e1.
241. Lee C, Longo V. Dietary restriction with and without caloric restriction for healthy aging. F1000Res. 2016;5.
242. de Siqueira KC, de Lima FM, Lima FS, Taki MS, da Cunha CF, de Lima Reis SR, et al. miR-124a expression contributes to the monophasic pattern of insulin secretion in islets from pregnant rats submitted to a low-protein diet. Eur J Nutr. 2017.
243. Mercken EM, Majounie E, Ding J, Guo R, Kim J, Bernier M, et al. Age-associated miRNA alterations in skeletal muscle from rhesus monkeys reversed by caloric restriction. Aging (Albany NY). 2013;5(9):692-703.
244. Olivo-Marston SE, Hursting SD, Perkins SN, Schetter A, Khan M, Croce C, et al. Effects of calorie restriction and diet-induced obesity on murine colon carcinogenesis, growth and inflammatory factors, and microRNA expression. PLoS One. 2014;9(4):e94765.
245. Jin L, Lim M, Zhao S, Sano Y, Simone BA, Savage JE, et al. The metastatic potential of triple-negative breast cancer is decreased via caloric restriction-mediated reduction of the miR-17~92 cluster. Breast Cancer Res Treat. 2014;146(1):41-50.
246. Zhang L, Hou D, Chen X, Li D, Zhu L, Zhang Y, et al. Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA. Cell Res. 2012;22(1):107-26.
247. Yang J, Farmer LM, Agyekum AA, Elbaz-Younes I, Hirschi KD. Detection of an Abundant Plant-Based Small RNA in Healthy Consumers. PLoS One. 2015;10(9):e0137516.
248. Beatty M, Guduric-Fuchs J, Brown E, Bridgett S, Chakravarthy U, Hogg RE, et al. Small RNAs from plants, bacteria and fungi within the order Hypocreales are ubiquitous in human plasma. BMC genomics. 2014;15:933.
249. Chin AR, Fong MY, Somlo G, Wu J, Swiderski P, Wu X, et al. Cross-kingdom inhibition of breast cancer growth by plant miR159. Cell Res. 2016;26(2):217-28.
250. Lukasik A, Zielenkiewicz P. In silico identification of plant miRNAs in mammalian breast milk exosomes--a small step forward? PLoS One. 2014;9(6):e99963.
251. Dickinson B, Zhang Y, Petrick JS, Heck G, Ivashuta S, Marshall WS. Lack of detectable oral bioavailability of plant microRNAs after feeding in mice. Nat Biotechnol. 2013;31(11):965-7.
252. Witwer KW. Contamination or artifacts may explain reports of plant miRNAs in humans. J Nutr Biochem. 2015;26(12):1685.
253. Witwer KW, McAlexander MA, Queen SE, Adams RJ. Real-time quantitative PCR and droplet digital PCR for plant miRNAs in mammalian blood provide little evidence for general uptake of dietary miRNAs: Limited evidence for general uptake of dietary plant xenomiRs. RNA Biol. 2013;10(7):1080-6.
254. Csiszar A, Gautam T, Sosnowska D, Tarantini S, Banki E, Tucsek Z, et al. Caloric restriction confers persistent anti-oxidative, pro-angiogenic, and anti-inflammatory effects and promotes anti-

aging miRNA expression profile in cerebromicrovascular endothelial cells of aged rats. American journal of physiology Heart and circulatory physiology. 2014;307(3):H292-306.
255. Valls R-M, Farràs M, Pedret A, Fernández-Castillejo S, Catalán Ú, Romeu M, et al. Virgin olive oil enriched with its own phenolic compounds or complemented with thyme improves endothelial function: The potential role of plasmatic fat-soluble vitamins. A double blind, randomized, controlled, cross-over clinical trial. Journal of Functional Foods. 2017;28:285-92.
256. Ewing JA. Detecting alcoholism. The CAGE questionnaire. JAMA. 1984;252(14):1905-7.
257. Rossi S, Calabretta A, Tedeschi T, Sforza S, Arcioni S, Baldoni L, et al. Selective recognition of DNA from olive leaves and olive oil by PNA and modified-PNA microarrays. Artificial DNA, PNA & XNA. 2012;3(2):63-72.
258. Dweep H, Sticht C, Pandey P, Gretz N. miRWalk--database: prediction of possible miRNA binding sites by "walking" the genes of three genomes. J Biomed Inform. 2011;44(5):839-47.
259. Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ. miRBase: tools for microRNA genomics. Nucleic Acids Res. 2008;36(Database issue):D154-8.
260. Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 2004;64(15):5245-50.
261. Marçais G, Kingsford C. A fast, lock-free approach for efficient parallel counting of occurrences of k-mers. Bioinformatics. 2011;27(6):764-70.
262. Medina I, Carbonell J, Pulido L, Madeira SC, Goetz S, Conesa A, et al. Babelomics: an integrative platform for the analysis of transcriptomics, proteomics and genomic data with advanced functional profiling. Nucleic Acids Res. 2010;38(Web Server issue):W210-3.
263. Szklarczyk D, Morris JH, Cook H, Kuhn M, Wyder S, Simonovic M, et al. The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. Nucleic Acids Res. 2017;45(D1):D362-D8.
264. Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, et al. TM4: a free, open-source system for microarray data management and analysis. Biotechniques. 2003;34(2):374-8.
265. Chen X, Gao C, Li H, Huang L, Sun Q, Dong Y, et al. Identification and characterization of microRNAs in raw milk during different periods of lactation, commercial fluid, and powdered milk products. Cell Res. 2010;20(10):1128-37.
266. Hata T, Murakami K, Nakatani H, Yamamoto Y, Matsuda T, Aoki N. Isolation of bovine milk-derived microvesicles carrying mRNAs and microRNAs. Biochemical and biophysical research communications. 2010;396(2):528-33.
267. Title AC, Denzler R, Stoffel M. Uptake and Function Studies of Maternal Milk-derived MicroRNAs. J Biol Chem. 2015;290(39):23680-91.
268. Alsaweed M, Hartmann PE, Geddes DT, Kakulas F. MicroRNAs in Breastmilk and the Lactating Breast: Potential Immunoprotectors and Developmental Regulators for the Infant and the Mother. Int J Environ Res Public Health. 2015;12(11):13981-4020.
269. Donaire L, Pedrola L, Rosa ReL, Llave C. High-throughput sequencing of RNA silencing-associated small RNAs in olive (Olea europaea L.). PLoS One. 2011;6(11):e27916.
270. Payne GA, Bohlen HG, Dincer UD, Borbouse L, Tune JD. Periadventitial adipose tissue impairs coronary endothelial function via PKC-beta-dependent phosphorylation of nitric oxide synthase. Am J Physiol Heart Circ Physiol. 2009;297(1):H460-5.
271. Schimmack G, Schorpp K, Kutzner K, Gehring T, Brenke JK, Hadian K, et al. YOD1/TRAF6 association balances p62-dependent IL-1 signaling to NF-κB. Elife. 2017;6.
272. Rehage N, Davydova E, Conrad C, Behrens G, Maiser A, Stehklein JE, et al. Binding of NUFIP2 to Roquin promotes recognition and regulation of ICOS mRNA. Nat Commun. 2018;9(1):299.
273. Mo W, Zhang JT. Human ABCG2: structure, function, and its role in multidrug resistance. Int J Biochem Mol Biol. 2012;3(1):1-27.
274. Aaronson DS, Horvath CM. A road map for those who don't know JAK-STAT. Science. 2002;296(5573):1653-5.
275. Quifer-Rada P, Martínez-Huélamo M, Chiva-Blanch G, Jáuregui O, Estruch R, Lamuela-Raventós RM. Urinary isoxanthohumol is a specific and accurate biomarker of beer consumption. J Nutr. 2014;144(4):484-8.

276. Li CW, Wang WH, Chen BS. Investigating the specific core genetic-and-epigenetic networks of cellular mechanisms involved in human aging in peripheral blood mononuclear cells. Oncotarget. 2016;7(8):8556-79.
277. Kim JH, Lee BR, Choi ES, Lee KM, Choi SK, Cho JH, et al. Reverse Expression of Aging-Associated Molecules through Transfection of miRNAs to Aged Mice. Mol Ther Nucleic Acids. 2017;6:106-15.
278. Moreira A, Groß S, Kirchberger MC, Erdmann M, Schuler G, Heinzerling L. Senescence markers - predictive for response to checkpoint inhibitors? Int J Cancer. 2018.
279. Cura Daball P, Ventura Ferreira MS, Ammann S, Klemann C, Lorenz MR, Warthorst U, et al. CD57 identifies T cells with functional senescence before terminal differentiation and relative telomere shortening in patients with activated PI3 kinase delta syndrome. Immunol Cell Biol. 2018.
280. Ordovas J. Diet/genetic interactions and their effects on inflammatory markers. Nutr Rev. 2007;65(12 Pt 2):S203-7.
281. Snow JW, Hale AE, Isaacs SK, Baggish AL, Chan SY. Ineffective delivery of diet-derived microRNAs to recipient animal organisms. RNA Biol. 2013;10(7):1107-16.
282. Melnik BC, John SM, Schmitz G. Milk: an exosomal microRNA transmitter promoting thymic regulatory T cell maturation preventing the development of atopy? J Transl Med. 2014;12:43.
283. Luo Y, Wang P, Wang X, Wang Y, Mu Z, Li Q, et al. Detection of dietetically absorbed maize-derived microRNAs in pigs. Sci Rep. 2017;7(1):645.
284. Wang W, Hang C, Zhang Y, Chen M, Meng X, Cao Q, et al. Dietary miR-451 protects erythroid cells from oxidative stress via increasing the activity of Foxo3 pathway. Oncotarget. 2017;8(63):107109-24.
285. Huang H, Davis CD, Wang TTY. Extensive Degradation and Low Bioavailability of Orally Consumed Corn miRNAs in Mice. Nutrients. 2018;10(2).
286. Baier SR, Nguyen C, Xie F, Wood JR, Zempleni J. MicroRNAs are absorbed in biologically meaningful amounts from nutritionally relevant doses of cow milk and affect gene expression in peripheral blood mononuclear cells, HEK-293 kidney cell cultures, and mouse livers. J Nutr. 2014;144(10):1495-500.
287. Mestdagh P, Hartmann N, Baeriswyl L, Andreasen D, Bernard N, Chen C, et al. Evaluation of quantitative miRNA expression platforms in the microRNA quality control (miRQC) study. Nat Methods. 2014;11(8):809-15.
288. Tosar JP, Rovira C, Naya H, Cayota A. Mining of public sequencing databases supports a non-dietary origin for putative foreign miRNAs: underestimated effects of contamination in NGS. RNA. 2014;20(6):754-7.
289. Lusk RW. Diverse and widespread contamination evident in the unmapped depths of high throughput sequencing data. PLoS One. 2014;9(10):e110808.
290. Piroddi M, Albini A, Fabiani R, Giovannelli L, Luceri C, Natella F, et al. Nutrigenomics of extra-virgin olive oil: A review. Biofactors. 2017;43(1):17-41.
291. Brennan E, Wang B, McClelland A, Mohan M, Marai M, Beuscart O, et al. Protective Effect of let-7 miRNA Family in Regulating Inflammation in Diabetes-Associated Atherosclerosis. Diabetes. 2017;66(8):2266-77.
292. Aparicio-Soto M, Montserrat-de la Paz S, Sanchez-Hidalgo M, Cardeno A, Bermudez B, Muriana FJG, et al. Virgin olive oil and its phenol fraction modulate monocyte/macrophage functionality: a potential therapeutic strategy in the treatment of systemic lupus erythematosus. Br J Nutr. 2018:1-12.
293. Pereira-da-Silva T, Coutinho Cruz M, Carrusca C, Cruz Ferreira R, Napoleão P, Mota Carmo M. Circulating microRNA profiles in different arterial territories of stable atherosclerotic disease: a systematic review. Am J Cardiovasc Dis. 2018;8(1):1-13.
294. Huang S, Lv Z, Guo Y, Li L, Zhang Y, Zhou L, et al. Identification of Blood Let-7e-5p as a Biomarker for Ischemic Stroke. PLoS One. 2016;11(10):e0163951.
295. Li S, Zhu J, Zhang W, Chen Y, Zhang K, Popescu LM, et al. Signature microRNA expression profile of essential hypertension and its novel link to human cytomegalovirus infection. Circulation. 2011;124(2):175-84.

296. Krause BJ, Carrasco-Wong I, Dominguez A, Arnaiz P, Farías M, Barja S, et al. Micro-RNAs Let7e and 126 in Plasma as Markers of Metabolic Dysfunction in 10 to 12 Years Old Children. PLoS One. 2015;10(6):e0128140.
297. Zhou P, Ma L, Zhou J, Jiang M, Rao E, Zhao Y, et al. miR-17-92 plays an oncogenic role and conveys chemo-resistance to cisplatin in human prostate cancer cells. Int J Oncol. 2016;48(4):1737-48.
298. Jiang C, Bi C, Jiang X, Tian T, Huang X, Wang C, et al. The miR-17~92 cluster activates mTORC1 in mantle cell lymphoma by targeting multiple regulators in the STK11/AMPK/TSC/mTOR pathway. Br J Haematol. 2018.
299. Doebele C, Bonauer A, Fischer A, Scholz A, Reiss Y, Urbich C, et al. Members of the microRNA-17-92 cluster exhibit a cell-intrinsic antiangiogenic function in endothelial cells. Blood. 2010;115(23):4944-50.
300. Bonauer A, Carmona G, Iwasaki M, Mione M, Koyanagi M, Fischer A, et al. MicroRNA-92a controls angiogenesis and functional recovery of ischemic tissues in mice. Science. 2009;324(5935):1710-3.
301. Fichtlscherer S, De Rosa S, Fox H, Schwietz T, Fischer A, Liebetrau C, et al. Circulating microRNAs in patients with coronary artery disease. Circulation research. 2010;107(5):677-84.
302. Pheiffer C, Dias S, Rheeder P, Adam S. Decreased Expression of Circulating miR-20a-5p in South African Women with Gestational Diabetes Mellitus. Mol Diagn Ther. 2018;22(3):345-52.
303. Margolis LM, Lessard SJ, Ezzyat Y, Fielding RA, Rivas DA. Circulating MicroRNA Are Predictive of Aging and Acute Adaptive Response to Resistance Exercise in Men. J Gerontol A Biol Sci Med Sci. 2017;72(10):1319-26.
304. Xiao D, Zhou T, Fu Y, Wang R, Zhang H, Li M, et al. MicroRNA-17 impairs glucose metabolism in insulin-resistant skeletal muscle via repressing glucose transporter 4 expression. Eur J Pharmacol. 2018.
305. Keita H, Ramírez-San Juan E, Paniagua-Castro N, Garduño-Siciliano L, Quevedo L. The long-term ingestion of a diet high in extra virgin olive oil produces obesity and insulin resistance but protects endothelial function in rats: a preliminary study. Diabetol Metab Syndr. 2013;5(1):53.
306. Estruch R, Martínez-González MA, Corella D, Salas-Salvadó J, Ruiz-Gutiérrez V, Covas MI, et al. Effects of a Mediterranean-style diet on cardiovascular risk factors: a randomized trial. Ann Intern Med. 2006;145(1):1-11.
307. He F, Lv P, Zhao X, Wang X, Ma X, Meng W, et al. Predictive value of circulating miR-328 and miR-134 for acute myocardial infarction. Mol Cell Biochem. 2014;394(1-2):137-44.
308. Lu Y, Zhang Y, Wang N, Pan Z, Gao X, Zhang F, et al. MicroRNA-328 contributes to adverse electrical remodeling in atrial fibrillation. Circulation. 2010;122(23):2378-87.
309. Klenke S, Eul S, Peters J, Neumann T, Adamzik M, Frey UH. Circulating miR-192 is a prognostic marker in patients with ischemic cardiomyopathy. Future Cardiol. 2018;14(4):283-9.
310. Jones A, Danielson KM, Benton MC, Ziegler O, Shah R, Stubbs RS, et al. miRNA Signatures of Insulin Resistance in Obesity. Obesity (Silver Spring). 2017;25(10):1734-44.
311. Fang Y, Shi C, Manduchi E, Civelek M, Davies PF. MicroRNA-10a regulation of proinflammatory phenotype in athero-susceptible endothelium in vivo and in vitro. Proc Natl Acad Sci U S A. 2010;107(30):13450-5.
312. Simionescu N, Niculescu LS, Sanda GM, Margina D, Sima AV. Analysis of circulating microRNAs that are specifically increased in hyperlipidemic and/or hyperglycemic sera. Mol Biol Rep. 2014;41(9):5765-73.
313. Wongwarawipat T, Papageorgiou N, Bertsias D, Siasos G, Tousoulis D. Olive Oil-related Anti-inflammatory Effects on Atherosclerosis: Potential Clinical Implications. Endocr Metab Immune Disord Drug Targets. 2018;18(1):51-62.
314. Casas R, Estruch R, Sacanella E. The Protective Effects of Extra Virgin Olive Oil on Immune-mediated Inflammatory Responses. Endocr Metab Immune Disord Drug Targets. 2018;18(1):23-35.
315. Violi F, Loffredo L, Pignatelli P, Angelico F, Bartimoccia S, Nocella C, et al. Extra virgin olive oil use is associated with improved post-prandial blood glucose and LDL cholesterol in healthy subjects. Nutr Diabetes. 2015;5:e172.

316. Olivieri F, Spazzafumo L, Santini G, Lazzarini R, Albertini MC, Rippo MR, et al. Age-related differences in the expression of circulating microRNAs: miR-21 as a new circulating marker of inflammaging. Mechanisms of ageing and development. 2012;133(11-12):675-85.
317. Ameling S, Kacprowski T, Chilukoti RK, Malsch C, Liebscher V, Suhre K, et al. Associations of circulating plasma microRNAs with age, body mass index and sex in a population-based study. BMC Med Genomics. 2015;8:61.
318. Jakob P, Kacprowski T, Briand-Schumacher S, Heg D, Klingenberg R, Stähli BE, et al. Profiling and validation of circulating microRNAs for cardiovascular events in patients presenting with ST-segment elevation myocardial infarction. Eur Heart J. 2017;38(7):511-5.
319. Martello A, Mellis D, Meloni M, Howarth A, Ebner D, Caporali A, et al. Phenotypic miRNA Screen Identifies miR-26b to Promote the Growth and Survival of Endothelial Cells. Mol Ther Nucleic Acids. 2018;13:29-43.
320. Kaneto CM, Nascimento JS, Moreira MCR, Ludovico ND, Santana AP, Silva RAA, et al. MicroRNA profiling identifies miR-7-5p and miR-26b-5p as differentially expressed in hypertensive patients with left ventricular hypertrophy. Braz J Med Biol Res. 2017;50(12):e6211.
321. Horibata Y, Sugimoto H. StarD7 mediates the intracellular trafficking of phosphatidylcholine to mitochondria. J Biol Chem. 2010;285(10):7358-65.
322. Flores-Martín J, Reyna L, Ridano ME, Panzetta-Dutari GM, Genti-Raimondi S. Suppression of StarD7 promotes endoplasmic reticulum stress and induces ROS production. Free Radic Biol Med. 2016;99:286-95.
323. Salminen A, Kaarniranta K. AMP-activated protein kinase (AMPK) controls the aging process via an integrated signaling network. Ageing Res Rev. 2012;11(2):230-41.
324. Tain YL, Lee WC, Wu KLH, Leu S, Chan JYH. Maternal High Fructose Intake Increases the Vulnerability to Post-Weaning High-Fat Diet-Induced Programmed Hypertension in Male Offspring. Nutrients. 2018;10(1).
325. Zhao M, Li Y, Yao H, Dou L, Zhang S, Zhao Q, et al. Sex-specific Alterations in Serology and the Expression of Liver FATP4 Protein in Offspring Exposed to High-Fat Diet during Pregnancy and/or Lactation. Lipids. 2018;53(3):301-11.
326. Tsai PH, Cheng CH, Lin CY, Huang YT, Lee LT, Kandaswami CC, et al. Dietary Flavonoids Luteolin and Quercetin Suppressed Cancer Stem Cell Properties and Metastatic Potential of Isolated Prostate Cancer Cells. Anticancer Res. 2016;36(12):6367-80.
327. Rubió L, Valls RM, Macià A, Pedret A, Giralt M, Romero MP, et al. Impact of olive oil phenolic concentration on human plasmatic phenolic metabolites. Food Chem. 2012;135(4):2922-9.
328. Pedret A, Fernández-Castillejo S, Valls RM, Catalán Ú, Rubió L, Romeu M, et al. Cardiovascular Benefits of Phenol-Enriched Virgin Olive Oils: New Insights from the Virgin Olive Oil and HDL Functionality (VOHF) Study. Mol Nutr Food Res. 2018;62(16):e1800456.
329. Halliwell B. Dietary polyphenols: good, bad, or indifferent for your health? Cardiovasc Res. 2007;73(2):341-7.
330. Long LH, Clement MV, Halliwell B. Artifacts in cell culture: rapid generation of hydrogen peroxide on addition of (-)-epigallocatechin, (-)-epigallocatechin gallate, (+)-catechin, and quercetin to commonly used cell culture media. Biochem Biophys Res Commun. 2000;273(1):50-3.
331. Hackl M, Brunner S, Fortschegger K, Schreiner C, Micutkova L, Muck C, et al. miR-17, miR-19b, miR-20a, and miR-106a are down-regulated in human aging. Aging cell. 2010;9(2):291-6.
332. Possemiers S, Bolca S, Grootaert C, Heyerick A, Decroos K, Dhooge W, et al. The prenylflavonoid isoxanthohumol from hops (Humulus lupulus L.) is activated into the potent phytoestrogen 8-prenylnaringenin in vitro and in the human intestine. J Nutr. 2006;136(7):1862-7.
333. Bolca S, Possemiers S, Maervoet V, Huybrechts I, Heyerick A, Vervarcke S, et al. Microbial and dietary factors associated with the 8-prenylnaringenin producer phenotype: a dietary intervention trial with fifty healthy post-menopausal Caucasian women. Br J Nutr. 2007;98(5):950-9.
334. Martinez SE, Davies NM. Enantiospecific pharmacokinetics of isoxanthohumol and its metabolite 8-prenylnaringenin in the rat. Mol Nutr Food Res. 2015;59(9):1674-89.
335. Luan YY, Yao YM. The Clinical Significance and Potential Role of C-Reactive Protein in Chronic Inflammatory and Neurodegenerative Diseases. Front Immunol. 2018;9:1302.

336. Ano Y, Dohata A, Taniguchi Y, Hoshi A, Uchida K, Takashima A, et al. Iso-α-acids, Bitter Components of Beer, Prevent Inflammation and Cognitive Decline Induced in a Mouse Model of Alzheimer's Disease. J Biol Chem. 2017;292(9):3720-8.
337. Negrão R, Costa R, Duarte D, Taveira Gomes T, Mendanha M, Moura L, et al. Angiogenesis and inflammation signaling are targets of beer polyphenols on vascular cells. J Cell Biochem. 2010;111(5):1270-9.
338. Scherr J, Nieman DC, Schuster T, Habermann J, Rank M, Braun S, et al. Nonalcoholic beer reduces inflammation and incidence of respiratory tract illness. Med Sci Sports Exerc. 2012;44(1):18-26.
339. Martínez Alvarez JR, Bellés VV, López-Jaén AB, Marín AV, Codoñer-Franch P. Effects of alcohol-free beer on lipid profile and parameters of oxidative stress and inflammation in elderly women. Nutrition. 2009;25(2):182-7.
340. Padro T, Muñoz-García N, Vilahur G, Chagas P, Deyà A, Antonijoan RM, et al. Moderate Beer Intake and Cardiovascular Health in Overweight Individuals. Nutrients. 2018;10(9).
341. Xiao J, Yu W, Hu K, Li M, Chen J, Li Z. miR-92a promotes tumor growth of osteosarcoma by targeting PTEN/AKT signaling pathway. Oncol Rep. 2017;37(4):2513-21.
342. Ye Y, Perez-Polo JR, Qian J, Birnbaum Y. The role of microRNA in modulating myocardial ischemia-reperfusion injury. Physiol Genomics. 2011;43(10):534-42.
343. Schneider A, Dhahbi JM, Atamna H, Clark JP, Colman RJ, Anderson RM. Caloric restriction impacts plasma microRNAs in rhesus monkeys. Aging cell. 2017;16(5):1200-3.
344. Liu R, Liao J, Yang M, Shi Y, Peng Y, Wang Y, et al. Circulating miR-155 expression in plasma: a potential biomarker for early diagnosis of esophageal cancer in humans. J Toxicol Environ Health A. 2012;75(18):1154-62.
345. Hulsmans M, Holvoet P. MicroRNA-containing microvesicles regulating inflammation in association with atherosclerotic disease. Cardiovasc Res. 2013;100(1):7-18.
346. Elgheznawy A, Shi L, Hu J, Wittig I, Laban H, Pircher J, et al. Dicer cleavage by calpain determines platelet microRNA levels and function in diabetes. Circ Res. 2015;117(2):157-65.
347. Zampetaki A, Kiechl S, Drozdov I, Willeit P, Mayr U, Prokopi M, et al. Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. Circ Res. 2010;107(6):810-7.
348. Paraskevi A, Theodoropoulos G, Papaconstantinou I, Mantzaris G, Nikiteas N, Gazouli M. Circulating MicroRNA in inflammatory bowel disease. J Crohns Colitis. 2012;6(9):900-4.
349. Tian ZQ, Jiang H, Lu ZB. MiR-320 regulates cardiomyocyte apoptosis induced by ischemia-reperfusion injury by targeting AKIP1. Cell Mol Biol Lett. 2018;23:41.
350. Ren XP, Wu J, Wang X, Sartor MA, Jones K, Qian J, et al. MicroRNA-320 is involved in the regulation of cardiac ischemia/reperfusion injury by targeting heat-shock protein 20. Circulation. 2009;119(17):2357-66.
351. Trajkovski M, Hausser J, Soutschek J, Bhat B, Akin A, Zavolan M, et al. MicroRNAs 103 and 107 regulate insulin sensitivity. Nature. 2011;474(7353):649-53.
352. Arpón A, Riezu-Boj JI, Milagro FI, Marti A, Razquin C, Martínez-González MA, et al. Adherence to Mediterranean diet is associated with methylation changes in inflammation-related genes in peripheral blood cells. J Physiol Biochem. 2016;73(3):445-55.
353. Zhu D, Pan C, Li L, Bian Z, Lv Z, Shi L, et al. MicroRNA-17/20a/106a modulate macrophage inflammatory responses through targeting signal-regulatory protein α. J Allergy Clin Immunol. 2013;132(2):426-36.e8.
354. Liang B, Wang X, Song X, Bai R, Yang H, Yang Z, et al. MicroRNA-20a/b regulates cholesterol efflux through post-transcriptional repression of ATP-binding cassette transporter A1. Biochim Biophys Acta. 2017;1862(9):929-38.
355. de Gaetano G, Costanzo S, Di Castelnuovo A, Badimon L, Bejko D, Alkerwi A, et al. Effects of moderate beer consumption on health and disease: A consensus document. Nutr Metab Cardiovasc Dis. 2016;26(6):443-67.
356. Collaborators GA. Alcohol use and burden for 195 countries and territories, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet. 2018;392(10152):1015-35.

357. Zhang L, Huang C, Guo Y, Gou X, Hinsdale M, Lloyd P, et al. MicroRNA-26b Modulates the NF-κB Pathway in Alveolar Macrophages by Regulating PTEN. J Immunol. 2015;195(11):5404-14.
358. Chiva-Blanch G, Magraner E, Condines X, Valderas-Martínez P, Roth I, Arranz S, et al. Effects of alcohol and polyphenols from beer on atherosclerotic biomarkers in high cardiovascular risk men: a randomized feeding trial. Nutr Metab Cardiovasc Dis. 2015;25(1):36-45.
359. Li S, Sun W, Zheng H, Tian F. Microrna-145 accelerates the inflammatory reaction through activation of NF-κB signaling in atherosclerosis cells and mice. Biomed Pharmacother. 2018;103:851-7.
360. Zhang Y, Zhang M, Zhong M, Suo Q, Lv K. Expression profiles of miRNAs in polarized macrophages. Int J Mol Med. 2013;31(4):797-802.
361. Li R, Shen Q, Wu N, He M, Liu N, Huang J, et al. MiR-145 improves macrophage-mediated inflammation through targeting Arf6. Endocrine. 2018;60(1):73-82.
362. Yuan X, Berg N, Lee JW, Le TT, Neudecker V, Jing N, et al. MicroRNA miR-223 as regulator of innate immunity. J Leukoc Biol. 2018;104(3):515-24.
363. Zhang N, Fu L, Bu Y, Yao Y, Wang Y. Downregulated expression of miR-223 promotes Toll-like receptor-activated inflammatory responses in macrophages by targeting RhoB. Mol Immunol. 2017;91:42-8.
364. Chen Q, Wang H, Liu Y, Song Y, Lai L, Han Q, et al. Inducible microRNA-223 down-regulation promotes TLR-triggered IL-6 and IL-1β production in macrophages by targeting STAT3. PLoS One. 2012;7(8):e42971.
365. Li M, He Y, Zhou Z, Ramirez T, Gao Y, Ross RA, et al. MicroRNA-223 ameliorates alcoholic liver injury by inhibiting the IL-6-p47. Gut. 2017;66(4):705-15.
366. Androulidaki A, Iliopoulos D, Arranz A, Doxaki C, Schworer S, Zacharioudaki V, et al. The kinase Akt1 controls macrophage response to lipopolysaccharide by regulating microRNAs. Immunity. 2009;31(2):220-31.
367. Chen X, Liang H, Zhang J, Zen K, Zhang CY. Secreted microRNAs: a new form of intercellular communication. Trends Cell Biol. 2012;22(3):125-32.
368. Wei G, Yi S, Yong D, Shaozhuang L, Guangyong Z, Sanyuan H. miR-320 mediates diabetes amelioration after duodenal-jejunal bypass via targeting adipoR1. Surg Obes Relat Dis. 2018;14(7):960-71.
369. Zheng J, Xiao X, Zhang Q, Wang T, Yu M, Xu J. Maternal Low-Protein Diet Modulates Glucose Metabolism and Hepatic MicroRNAs Expression in the Early Life of Offspring †. Nutrients. 2017;9(3).
370. Paim LR, Schreiber R, de Rossi G, Matos-Souza JR, Costa E Silva A, Calegari DR, et al. Circulating microRNAs, vascular risk and physical activity in spinal cord-injured subjects. J Neurotrauma. 2018.
371. Sun X, Sit A, Feinberg MW. Role of miR-181 family in regulating vascular inflammation and immunity. Trends Cardiovasc Med. 2014;24(3):105-12.
372. Sharma H, Chinnappan M, Agarwal S, Dalvi P, Gunewardena S, O'Brien-Ladner A, et al. Macrophage-derived extracellular vesicles mediate smooth muscle hyperplasia: role of altered miRNA cargo in response to HIV infection and substance abuse. FASEB J. 2018;32(9):5174-85.
373. Wang Y, Zhang X, Tang W, Lin Z, Xu L, Dong R, et al. miR-130a upregulates mTOR pathway by targeting TSC1 and is transactivated by NF-κB in high-grade serous ovarian carcinoma. Cell Death Differ. 2017;24(12):2089-100.
374. Su S, Zhao Q, He C, Huang D, Liu J, Chen F, et al. miR-142-5p and miR-130a-3p are regulated by IL-4 and IL-13 and control profibrogenic macrophage program. Nat Commun. 2015;6:8523.
375. Chen C, Yang S, Li H, Yin Z, Fan J, Zhao Y, et al. Mir30c Is Involved in Diabetic Cardiomyopathy through Regulation of Cardiac Autophagy via BECN1. Mol Ther Nucleic Acids. 2017;7:127-39.
376. Horikoshi M, Hara K, Ohashi J, Miyake K, Tokunaga K, Ito C, et al. A polymorphism in the AMPKalpha2 subunit gene is associated with insulin resistance and type 2 diabetes in the Japanese population. Diabetes. 2006;55(4):919-23.
377. Ferreira GS, Pinto PR, Iborra RT, Del Bianco V, Santana MFM, Nakandakare ER, et al. Aerobic Exercise Training Selectively Changes Oxysterol Levels and Metabolism Reducing Cholesterol Accumulation in the Aorta of Dyslipidemic Mice. Front Physiol. 2017;8:644.

378. Chou JP, Effros RB. T cell replicative senescence in human aging. Current pharmaceutical design. 2013;19(9):1680-98.

PUBLICATIONS